

# Shake 'n Stack

## Hybridisation Oven

Operating and Maintenance Manual 7026240 Rev. 0



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**Models covered by this manual**

| <b>Models</b> | <b>Voltage</b> | <b>Includes</b>                         |
|---------------|----------------|-----------------------------------------|
| 6240          | 220            | Shaking platform, drip tray, rotisserie |
| 6241          | 110            | Shaking platform, drip tray, rotisserie |
| 6242          | 220            | Drip tray, rotisserie                   |
| 6243          | 110            | Drip tray, rotisserie                   |
| 6244          | 110            | Drip tray                               |
| 6245          | 110            | Shaking platform, drip tray             |

**Manual Number 7026240**

| 0          | 25036/OV-368   | 8/16/11     | New controller - Release 3 | ccs       |
|------------|----------------|-------------|----------------------------|-----------|
| <b>REV</b> | <b>ECR/ECN</b> | <b>DATE</b> | <b>DESCRIPTION</b>         | <b>By</b> |



**Important** Read this instruction manual. Failure to read, understand and follow the instructions in this manual may result in damage to the unit, injury to operating personnel, and poor equipment performance. s

**Caution** All internal adjustments and maintenance must be performed by qualified service personnel. s

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- 4 Always use the proper protective equipment (clothing, gloves, goggles, etc.)
- 4 Always dissipate extreme cold or heat and wear protective clothing.
- 4 Always follow good hygiene practices.
- 4 Each individual is responsible for his or her own safety.

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## Section 1 Introduction

The Thermo system of Hybridising in bottles is considered by many molecular biologists to be the best method for conducting Hybridisations with Southern, Northern, Dot, Slot or Colony Blots. Additional information, including detailed protocols, is contained in the included Hybridisation Guide.

Hybridising in bottles means that probe volumes may be significantly reduced compared to experiments performed in conventional systems, and the continual movement of the probe across the surface of the membrane results in very efficient hybridisation reactions.

Protection from exposure to radiation is provided by the heavy walled borosilicate glass bottles.

**Warning** Based on tests, these bottles block almost 100% of BETA radiation emissions of a 1,000 pCi P-32 source. However, users should observe precautions due to induced X-ray phenomenon (Bremsstrahlung) occurring, depending on the type(s) of radionuclides used. s

The Hybridisation Oven itself provides additional shielding. In the event of a spillage within the Oven, the stainless steel drip tray will contain up to 6.8 fl. oz. (200ml) of liquid.

**Note** If the Shake 'n Stack is not used as specified in this manual, the protection provided by the equipment may be impaired. s

### Oven Temperature Monitoring

All Ovens are calibrated by matching the thermistor temperature read-out, seen on the LCD display, to the actual measurement of distilled water contained within a Hybridisation Bottle as it rotates. The temperature measuring device used inside the calibration bottle is traceable to national standards.

This calibration method ensures all experiments using the rotisserie meet our temperature accuracy specification of  $\pm 1^{\circ}\text{C}$  and indeed, due to the rotisserie action, uniformity within the bottle is  $\pm 0.25^{\circ}\text{C}$ . However, it is important to note that the temperature inside the oven chamber will vary from point to point and therefore, the temperature of objects/vessels, which are not placed in the rotisserie, will vary from that which is displayed. Refer to “Setting the Required Temperature” for recommendations on temperature setting when using shaking platforms.

## Oven Temperature Monitoring (cont'd)

The Shake 'n Stack is designed to be used with Thermo's hybridisation bottle. These high quality leak-proof containers can be used at temperatures up to 70°C (for catalogue numbers, see Section 8).

Bottles should not be used above 70°C without taking the precaution of releasing the pressure formed by the release of gases from solutions as they are warmed. Simply loosening, then retightening the bottle cap at approximately 60°C should relieve the pressure.

**Caution** Dropping or over-tightening lids can cause cracking and failure. Lids should be inspected prior to each use. s

**Caution** Always wear gloves when handling the bottle and use the bottle gripper provided (see Appendix II for full instructions on Bottle Care). s

Finally, the Shake 'n Stack Oven is designed for reliability and ease of maintenance. The rotisserie, shaking platform and drip tray can be easily removed for cleaning.

## Safety Precautions

### Supply

Power rating: .....250W

Frequency: .....50/60Hz

Fuse rating: .....110/120V: 6.3A type T

.....220/240V: 1.6A type T

Ingress Protection Rating .....IP22

Rotisserie speed control PCB fuse: .....500mA

**Note** This item should not be serviced by the customer. s

Working Environment: .....15°C to 25°C

**Caution** Care should be taken when lifting the Oven. It is advisable that help is sought when removing an Oven from its packaging and when stacking Ovens for use. s

**Caution** During operation, caution should be taken with moving parts that are accessible when the Oven door is open. s

Thermo offers full service and technical support for all its products. Alternatively, contact your local authorised distributor.

## Section 2 Unpacking and Installation

### Shake 'n Stack Oven

4 Leveling Feet

Shaking Tray (Models 6240, 6241, 6245)

Shaking Tray Drive Arm Assembly (Models 6240, 6241, 6245)

Shaking Tray Bracket (Models 6240, 6241, 6245)

Power Cable

Drip Tray

**Note** If any item is missing or damaged, contact Thermo or an authorised Thermo distributor. s

**Warning** If it is intended to use the Oven with radioactive isotopes, the oven must be located in a designated Radiation Area. Radiation safety procedures must be followed at all times. s

- In the event of spillage, refer to Section 6 for guidance on cleaning and decontamination.
- All Hybridisation Oven standard rotisseries are designed to accept the long, medium or short Hybridisation Bottles.

Once unpacked, attach leveling feet to base, and position on a flat surface. Level the oven by adjusting the height of the leveling feet. Once leveled, the oven is ready for use. If adding to a stack of Shake 'n Stack Ovens (maximum of 3), attach feet and adjust height, then position feet in the 4 indents on the top of the supporting Shake 'n Stack Oven. Some additional adjustment of the feet may be necessary until the ovens sit in a stable position.

**Note** Shake 'n Stack Ovens have been designed to stack 3 units high. No attempt should be made to stack higher than this. s

Connect each individual oven to the mains supply using only the removable mains lead supplied. The mains lead should be fitted with a 10A fuse for both 110/120V and 220/240V regions. The oven is grounded via the mains lead and should only be connected to an grounded supply. (See “Technical Specifications”).

## Operation

The two switches found on the control panel operate the power and the rotisserie drive. The power switch is marked with a “1” and a “0”. By switching to the “1” position, the mains power is connected to the oven and the green section of the switch is exposed to indicate that the oven is “ON”. The switch to the right operates the rotisserie/shaker. The rotisserie/shaker switch cannot be operated without first turning on the power switch. To the left of the rotisserie/ shaker switch is a dial to adjust the rotisserie speed from 5-15 rpm and the shaker speed from 4-10 rpm.

Instructions to set up the shaking platform are in Section 4.

## Set the Required Temperature

The temperature controller has 4 buttons along the lower edge of the controller.

When the power is switched on, the display shows three dashes (---) as the controller goes through its internal self-tests, which are completed within 5 seconds. The display then shows the compensated temperature of the oven.

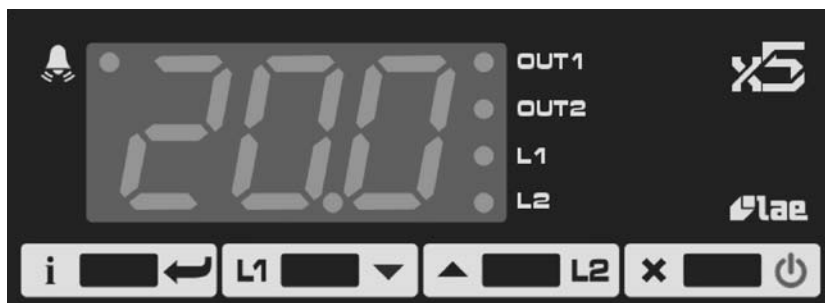


**Figure 2-1.** Temp Controller

To set the controller temperature, press and release the L1 button. The L1 LED will blink on-and-off, and the display will show 1SP for 1 second, then the present setpoint value. Press the up or down arrow button to change the desired setpoint within the oven operating range of 35°C to 85°C. Press the Enter button (↵) to store the new setpoint value, or press the X button to return to normal mode without saving the new setpoint value.

The warm up time for the oven varies according to the load and the ambient temperature. When first switching on the oven (no bottles installed), allow approximately one hour for the temperature to stabilise. Solutions should be pre-warmed to the required temperature to minimise sample stabilisation time.

## Set the Required Temp (continued)

For ease of use and reproducibility when hybridising in bottles, the temperature display is calibrated to show internal bottle temperature as opposed to oven air temperature. When the shaking platform is used, allowance must be made for the difference between the temperature shown on the display and the temperature achieved within a liquid-filled vessel on the shaker.

This difference can be calculated by filling the protocol specific vessel with enough distilled water to cover an immersible thermometer, and setting the oven to the desired product temperature. After enough time for the temperature to have stabilized, record the thermometer reading and subtract this value from the setpoint value. The remainder must be added to the setpoint value for execution of the actual protocol. For instance, a setpoint value of 55°C minus a thermometer reading of 58°C yields a remainder of -3°C which means that the adjusted setpoint value for this protocol will be 52°C. Repeat this process to verify that the thermometer reading is within +/-1°C of the setpoint value. This difference should be determined for each oven and/or shaker protocol.

The lowest target temperature which the oven can achieve is 8°C above ambient temperature. Avoid placing the unit in direct sunlight, or in any area where it is likely to be exposed to hazardous or volatile chemicals.

When positioning the Hybridisation Oven, ensure a gap of at least 2 in. (5cm) is maintained between the sides of the oven and any other equipment or object.

The oven may be located in a fume cabinet or similar chamber, provided the cabinet is not used to contain hazardous or volatile chemicals with low flash points.

Full details of Technical Specifications are given in Section 8.

**Note** If the Shake 'n Stack Oven is not used as specified in this manual, the protection provided by the equipment may be impaired.



## Section 3 Set Up the Shaking Platform

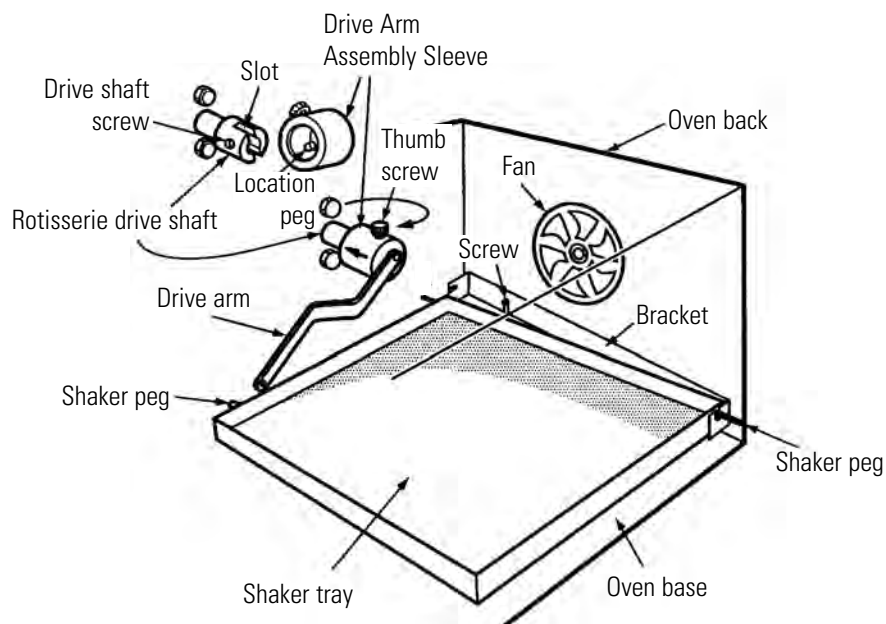


**Figure 1.** Shaking Tray Components

1. The shaker bracket is installed at the factory. If replacement should ever become necessary, instructions follow. Continue to Step 2.
- Loosen existing screws at the bottom of the oven back sufficiently to allow slots in bracket to slide down onto screw shafts. Retighten screws to secure back plate. See Figure 2.
2. Fit the rear right hand peg of tray into vertical slot at right of bracket, allowing tray to temporarily rest on oven base. Refer to Figure 3.

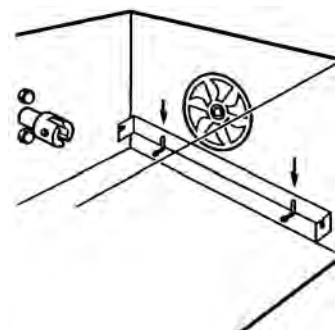


**Figure 2.** Replace Shaker Bracket

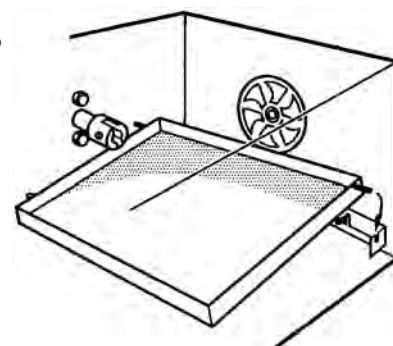


**Figure 3.** Insert Tray Peg into Slot

### Section 3

#### Set Up the Shaking Platform

3. Slide drive arm assembly sleeve onto rotisserie drive shaft, aligning location peg within drive arm sleeve into either slot on drive shaft, ensuring the sleeve is pushed fully onto shaft. Tighten using thumb screw.

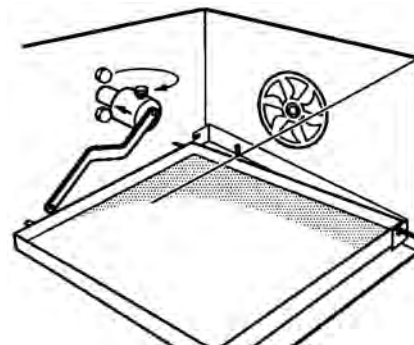


**Figure 4.** Slide Sleeve onto Shaft

4. Locate front left peg of tray into the hole at the end of the drive arm.

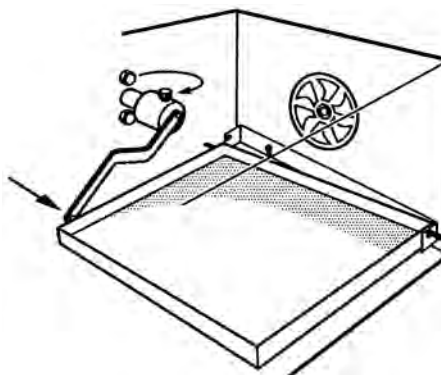


**Figure 5.** Tray Peg into Arm Hole

5. Slide the rear left peg into the horizontal slot on the left-hand side of bracket at the back of oven. Assembly is now complete and ready to operate.

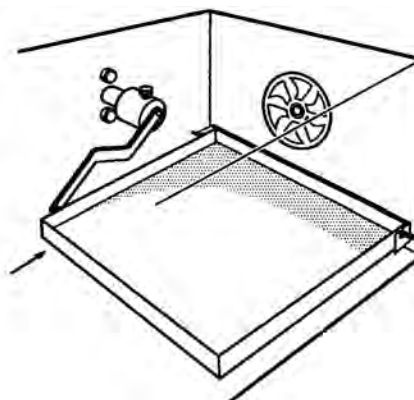


**Figure 6.** Rear Peg into Slot

## Section 4 Methodology of Hybridisation

The following procedures indicate how to use the Shake n' Stack Oven for hybridisation.

### Place Membranes in a Bottle

1. Place the bottles to be used into the oven during the warm-up period (approximately 1 hour).
2. Select a piece of support mesh appropriate for the size of the membrane. The recommended hybridisation mesh is supplied by Thermo (see parts list in Section 8).
3. Pre-wet the mesh and hybridisation membrane in a suitable tray containing 2 x SSPE (SSC). See Figure 4-1.
4. Ensuring that the hybridisation membrane exactly overlays the mesh, roll both up into a tight roll.

If more than one membrane is to be hybridised in a bottle, simply layer meshes and membranes as required before rolling. It is important that each membrane is separated from another by a layer of mesh. Up to five 0.8 x 0.8 in. (20 x 20mm) membranes can be hybridised in a single hybridisation bottle.

5. Place 0.34-0.51 fl. oz. (10-15ml) SSPE (SSC) into a hybridisation bottle and then insert the roll in such a way that the leading edge (inside the roll) and the trailing edge are positioned relative to each other as shown in Figure 4-3.
6. Place the bottle on a flat surface, then slowly unwind the membrane and mesh around the inside of the bottle by gently rocking and rolling the bottle along the surface. No air bubbles should be visible between the membrane and the bottle. If bubbles are present, the membrane should be removed and re-rolled.

The procedure should then be repeated more gently. Rock the bottle backwards and forwards to attach the first part of the membrane to the bottle. Then roll the bottle to unwind the mesh and membrane.

7. Continue until the membrane and mesh are fully unwound. The mesh ensures the probe will have access to all parts of the hybridisation membrane including those parts of the membrane which overlap.

## Insert Membranes in a Bottle

1. Assemble these items:
  - Shallow tray containing 2 x SSPE (SSC).
  - Mesh.
  - Membrane.

Roll up mesh and membrane (Figure 4-1).

2. Place 0.3-0.5 fl. oz. (10-15ml) of SSPE (SSC) into a bottle and then insert the roll centrally (Figure 4-2).

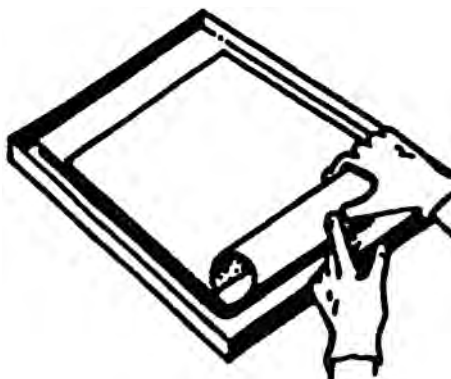


**Figure 1.** Roll Mesh and Membrane



**Figure 4-2.** Insert Roll

3. Secure cap and, holding bottle horizontally, roll to catch the trailing edge of the mesh; continue rolling in the same direction until coil of mesh and membrane is well positioned.

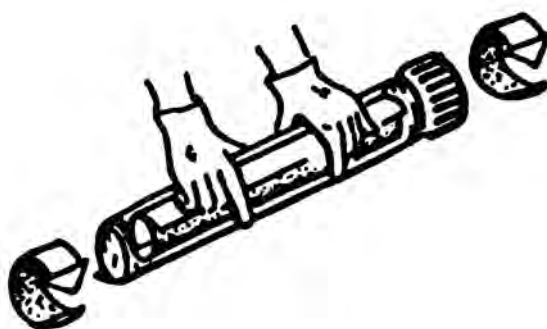


**Figure 4-3.** Rolling Direction of Bottle



**Figure 4-4.** Bottle in Oven Rotation

4. Pour out SSPE (SSC) and replace with pre-warmed pre-hybridisation buffer.
5. Place the bottle in the oven so that it rotates in the oven in the same direction, as it was unrolled in Step 3.

## Pre-Hybridisation

1. Once the membrane is in place in the bottle the SSPE (SSC) can be discarded and replaced with pre-hybridisation fluid.

This is easily done by removing the cap, pouring off the SSPE (SSC), then pouring in the pre-hybridisation fluid. All the solutions used during hybridisation should be pre-warmed before use. 0.34-0.51 fl. oz. (10-20ml) solution is recommended for medium bottles and 0.17-0.34 fl. oz. (5-10ml) for short bottles. Higher volumes will be required if there is more than one membrane in the bottle, e.g. 0.51-0.85 fl. oz. (15-25ml).

2. Replace the cap on the bottle and insert into the rotisserie.

## Hybridisation

1. Denature the purified probe by boiling for five minutes, then store on ice. If the volume to be used for hybridisation is substantially different than that used previously for hybridisation in bags or boxes, ensure that the quantity of the probe is adjusted accordingly to maintain the correct probe concentration. If this is not done, high background may result.
2. Remove the bottle from the Hybridisation Oven and unscrew the cap.
3. If the same buffer is to be used for hybridisation as for pre-hybridisation, simply pipette the probe into the pre-hybridisation fluid in the bottle. Take care to avoid pipetting the probe directly on to the membrane as this will result in hot spots. Alternatively, dilute the probe in pre-warmed hybridisation solution outside the bottle. Pour off the pre-hybridisation solution and replace with the probe solution.
4. Replace the cap and gently agitate the bottle to ensure an even distribution of the probe in the hybridisation solution.
5. Place the bottle back in the Hybridisation Oven, switch on the rotisserie and leave it to hybridise for the required time period.

## Washing

Two methods for washing follow.

### Washing in Hybridisation Bottles

1. Remove the bottle from the Oven.
2. Pour off the hybridisation fluid and then half fill the bottle with the first pre-warmed wash solution (approx. 3.38 fl. oz. [100ml]) and replace the cap.

**Note** Room temperature washing or washing with solutions which have not been pre-warmed may result in background problems (see Appendix I of the Hybridisation Guide at the end of this manual). s

3. Replace the bottle in the Oven and leave for the required time period.

If transferring from sandwich box or plastic bag methodology, the times and temperatures are usually those currently being used, approximately 20 minutes per wash step.

4. Repeat Steps 1-3 for each additional wash. The wash solutions, temperatures, etc., should be those recommended by the membrane manufacturer, or refer to the Hybridisation Guide.

**Note** All wash solutions should be pre-warmed for best results. s

### Washing in a Sandwich Box

Some scientists prefer to remove the membranes from the bottles and wash them all in one container. Washing may be performed very simply in the Shake 'n Stack on the shaking platform and is particularly useful when several membranes are being processed simultaneously.

1. Remove the hybridisation bottle from the oven. Pour off the hybridisation fluid.
2. Using a pair of forceps, carefully remove the membranes and place them in a suitable container with a lid (e.g. sandwich box or similar).
3. Using the forceps, gently unroll the membranes.

4. Add a sufficient volume of the pre-warmed first wash solution to totally immerse the membranes. Replace the lid and shake on the shaking platform in the Shake 'n Stack Oven at the required temperature for the specified time period.

**Note** Initial room temperature washes are not recommended and may result in subsequent background problems. s

5. Remove the first wash solution and replace it with an equal volume of the prewarmed second wash solution. Replace the lid and shake at the required temperature for the required time period.
6. Step 5 should be repeated for any subsequent wash. The washing protocol using the shaking platform will be identical to that used in the bottles.

**Note** Refer to Appendix I for additional information on Washing. s

In addition to bulk processing of blots during stringency washing steps, the shaking platform is ideal for performing several other stages of the blot generation and processing procedures. Three examples are given as follows:  
-

1. Pre-treatment of Gels prior to Nucleic Acid Transfer

Gentle agitation of the gel during pre-treatment steps is essential to prevent damage to the gel. Pre-treatment steps are usually performed at ambient temperature.

- (i) Depurination - 0.25M HCl 10 minutes
- (ii) Denaturation - 1.5M NaCl, 0.5M NaOH, 30 minutes
- (iii) Neutralisation - 1.5M NaCl, 0.5M Tris Cl pH 7.2, 30 minutes

2. Pre-washing of Filters prior to Colony Blot Hybridisation

Pre-washing is often advantageous to remove colony debris and thus reduce background noise when screening bacterial colonies by hybridisation. After lysing the bacterial colonies and fixing the DNA on the membrane, pre-wash in a solution of 2 x SSC/0.1% SDS at 65°C. Use several changes of buffer.

After several washes, residual colony debris may be removed by gently rubbing filter with a gloved finger.

## Section 4

### Methodology of Hybridisation

#### 3. Probe Stripping from Filters after Hybridisation

An example of probe stripping procedure is as follows: -

- a) Wash the filter at 65°C in 5mM Tris Cl pH8.0, 0.2mM EDTA, 0.1 x Denhardt's reagent for 1-2 hours.
- b) Check for residual activity by autoradiography, then re-probe.

## Section 5 Rotisseries

There are two kinds of rotisseries available; variable axis and tube rotisseries. Information follows.

### Variable Axis Rotisseries

The Shake 'n Stack is equipped with a variable axis rotisserie. This allows the user to alter the angle at which bottles can be held in the rotisserie. A small offset from horizontal of between 1-15° causes fluid to flow from end to end within the bottles during rotation (see Figure 5-1). This “wave” motion provides a more vigorous and active hybridisation, or washing stage, and can allow probe volumes to be reduced to as low as 0.07-0.17 fl. oz. (2-5ml). To achieve the optimum active wave conditions requires adjustment of the rotisserie angle. Note that a large angle offset from horizontal (15°) combined with a low probe volume (<0.03-0.07 fl. oz. [1-2ml]) can produce dry areas on the membrane and so should be avoided.

To alter the bottle angle:

1. Release the two securing screws visible on the front rotisserie wheel using a Phillips screwdriver.
2. Put the rotisserie into the oven and place a bottle in the rotisserie.
3. Rotate the free wheel to the desired angle.
4. Remove rotisserie and tighten the screw in the new position.

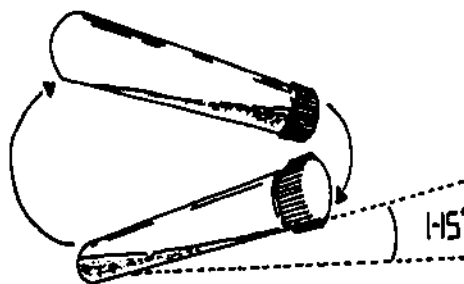


**Figure 5-1.** Variable Axis Rotation

## **50ml & 15ml Tube Rotisseries**

Rotisseries are available that can hold 0.5 fl. oz. (15ml) and/or 1.7 fl. oz. (50ml) disposable tubes. Details are given in Section 8.

Tubes should be inserted into the rotisseries by sliding the tube sideways into the rotisserie. Care should be taken not to push the tubes too firmly into the rotisseries, otherwise the tube will crack.

## Section 6 **Cleaning and Decontamination**

All items, which come into contact with a labeled probe, should be decontaminated before re-use. In many cases, a short rinse in water will be sufficient. In some instances, however, further decontamination procedures will be required.

### **Meshes**

The quantity of radioactivity adhering to the mesh can be minimised by leaving the mesh in with the membrane throughout the washing procedure. Any radioactivity remaining can then be stripped from the mesh by the following procedure:

1. Strip wash the mesh by incubating it in distilled water at 65°C in a shaking water bath for 15 minutes. Repeat.
2. If the mesh is still contaminated, soak it in a diluted solution, such as Decon 90, overnight.
3. Remove the detergent and proceed with two washes in distilled water for 10 minutes each.
4. If the mesh has been decontaminated, no further action is necessary. If, however, the mesh is still contaminated, it will be necessary to leave it to decontaminate in Decon 90 for a longer time period.
5. Meshes should be allowed to dry flat between uses.

## Bottles & Caps

The hybridisation bottles and caps can be decontaminated by the following procedure:

1. Soak the bottles and caps in a dilute detergent solution (Decon 90) overnight.
2. Remove the Decon 90 and rinse with distilled water.
3. If the bottles and caps have been decontaminated, no further action is necessary. If, however, they are still contaminated, gently scrub them with an abrasive cloth or brush and if necessary, leave to decontaminate in Decon 90 for a longer time period.

## Oven Interior/Shaking Platform

Stainless steel drip trays are provided with all Thermo ovens and are intended to contain spillages in the event of an accident. These, together with the stainless steel surfaces of the ovens and the shaking platform, can be decontaminated by wiping clean with Decon 90, followed by distilled water. No further action should be necessary.

## Rotisserie

The rotisserie may be cleaned with Decon 90 and distilled water. No further action should be necessary. Avoid alcohol or other organic solutions, which may affect the plastic surfaces.

**Caution** Before using any cleaning or decontamination method except those recommended by the manufacturer, users should check with the manufacturer that the proposed method will not damage the equipment. s

## Section 7 Use of Radioactive Probes

Thermo's Hybridisation Ovens have been tested with a variety of non-radioactive probes, e.g. the DIG Non-Radioactive Nucleic Acid labelling and detection system from Boehringer Mannheim.

Please refer to the Hybridisation Guide for guidelines on the use of non-radioactive systems.

**Warning** The Shake 'n' Stack Oven has not been designed for use with hazardous or volatile chemicals with low flash points such as dimethylformamide or chloroform. Thermo cannot accept responsibility for damages arising from such use. If in any doubt, contact a Thermo product specialist prior to use. s



## Section 8 Technical Specifications

### Temperature

|                          |              |
|--------------------------|--------------|
| Range                    | 35°C to 85°C |
| Selection                | Digital      |
| Display                  | Digital      |
| Monitor/Sensor           | Thermistor   |
| Uniformity (in bottle)   | ±0.25°C      |
| Accuracy (at 55°C)       | ±1.0°C       |
| Safety Over Temp Cut Out | 100°C        |

### Rotisserie

|                  |                                                                                  |
|------------------|----------------------------------------------------------------------------------|
| Rotisserie Speed | 5-15 rpm                                                                         |
| Capacity         | 10 long, medium & short bottles                                                  |
| Material         | Stainless steel shaft<br>Delrin plastic rotisserie wheels<br>Variable axis 0-15° |

### Shaker

|                         |                                                     |
|-------------------------|-----------------------------------------------------|
| Speed                   | 4-10 rpm                                            |
| Maximum Weight Capacity | 2.2 lb. (1kg)                                       |
| Max Load Dimensions     | 9.8"W x 7.1"H x 7.9"D<br>(250mmW x 180mmH x 200mmD) |
| Material                | Stainless steel                                     |

### Dimensions

|                 |                                                        |
|-----------------|--------------------------------------------------------|
| External        | 17.1"W x 16.7"H x 14.3"D<br>(435mmW x 425mmH x 363mmD) |
| Internal        | 13.6"W x 9.6"H x 9.5"D<br>(345mmW x 245mmH x 240mmD)   |
| Shipping Weight | 53 lb. (24kg)                                          |

**Features** .....Plastic Snap-fit Rotisserie  
.....Easy Set Temperature Controller

**Standard Accessories**

.....Shaking Tray Drive Arm Assembly -  
.....(Models 6240, 6241, 6244, 6245)  
.....Shaking Tray Bracket -  
.....(Models 6240, 6241, 6244, 6245)  
.....Power Cable  
.....1.4 in. (35mm) Rotisserie -  
.....(Models 6240, 6241, 6244, 6245)

**Ordering Information**

Shake 'n Stack

110V .....Model 6241

220V .....Model 6240

Includes shaking platform, drip tray and Delrin plastic rotisserie.

110V .....Model 6243

220V .....Model 6242

Includes drip tray and Delrin plastic rotisserie.

110V .....Model 6245

Includes shaking platform and drip tray.

110V .....Model 6244

Includes drip tray.

**Accessory Pack** .....P/N 222060

Includes 2 medium bottles and Bottle Gripper.

**Temp Controller Replacement Kit** ...P/N 192048

Includes temperature controller, PTC probe, gasket, and installation/  
calibration procedures.

## **Additional Accessories**

### **Rotisseries**

1.4 in. (35mm) Rotisserie . . . . .P/N 222032

Holds up to 10 long, medium & short bottles

2.76 in. (70mm) Rotisserie . . . . .P/N 222033

Holds up to 4 extra long bottles

Rotisseries to fit the Shake 'n Stack and Maxi 14 Hybridisation Ovens  
. . . . .P/N 222042

Holds up to 0.8 x .05 fl. oz. (25 x 15ml) tubes & 1.0 x 1.7 fl. oz.  
(30 x 50ml) tubes

Rotisserie to fit the Shake 'n Stack and Maxi 14 Hybridisation Ovens  
. . . . .P/N 222043

Holds up to 1.7 x 0.5 fl. oz. (50 x 15ml) tubes & 0.54 x 1.7 fl. oz.  
(16 x 50ml) tubes

Rotisserie to fit the Shake 'n Stack and Maxi 14 Hybridisation Ovens  
. . . . .P/N 222044

Holds up to 1.5 x 1.7 fl. oz. (44 x 50ml) tubes

**Shaking Platform** . . . . .P/N 222000

### **Hybridisation Bottles**

Extra-long bottle, 2.8 x 11.8 in. (70 x 300mm) . . .  
. . . . .P/N 110094

Long bottle, 1.4 x 11.8 in. (35 x 300mm) . . . . .  
. . . . .P/N 110113

Medium bottle, 1.4 x 9.8 in. (35 x 250mm) . . . . .  
. . . . .P/N 110115

Short bottle, 1.4 x 5.9 in. (35 x 150mm) . . . . .  
. . . . .P/N 110116

Bottle gripper: 11.8 in. (300mm) for extra-long bottles  
. . . . .P/N 222055

Bottle gripper: 11.8 in. (300mm) for long, medium bottles  
. . . . .P/N 222056

Bottle gripper: 5.9 in. (150mm) for short bottles  
. . . . .P/N 222057

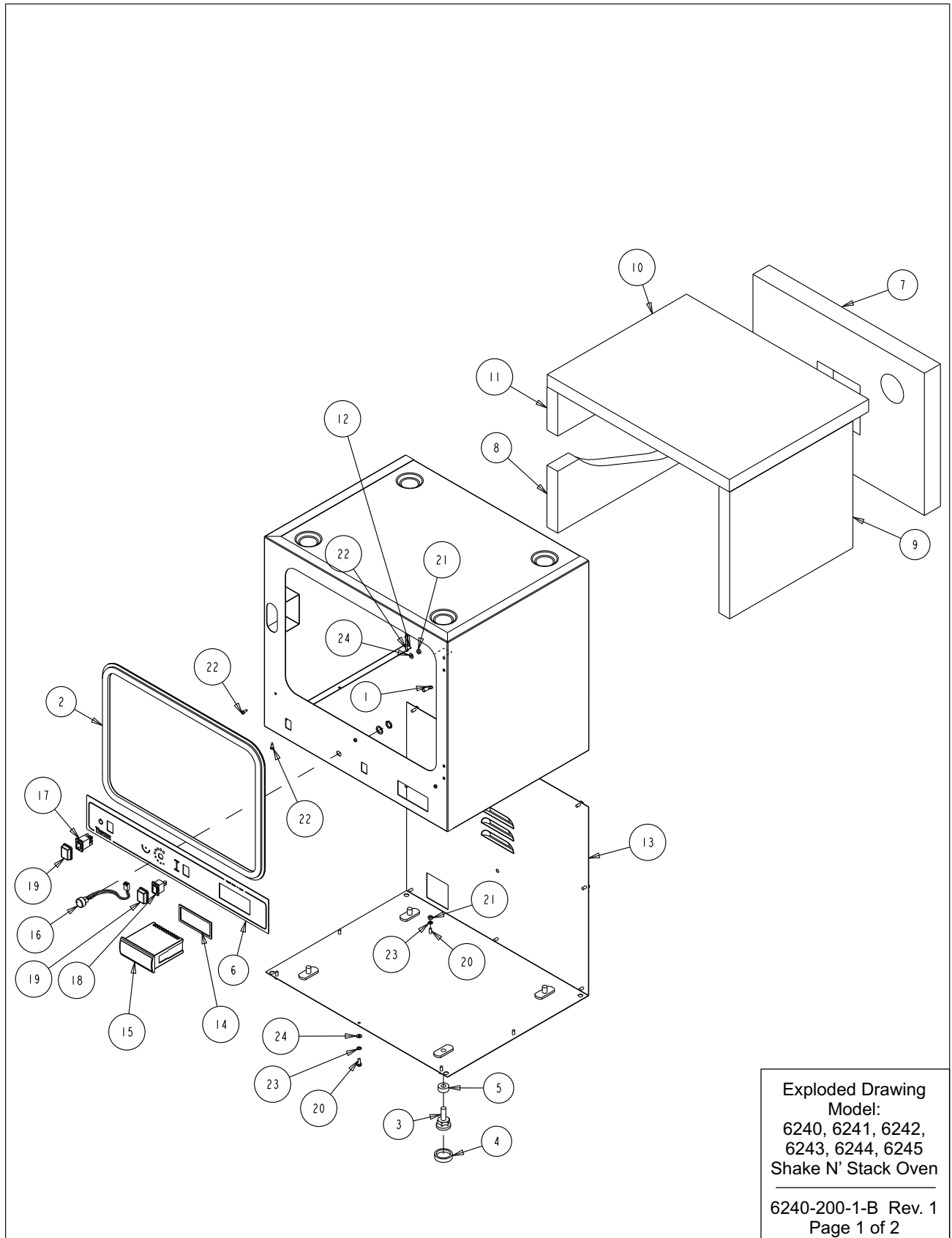
### **Nylon Mesh**

Five large mesh sheets, 9 x 9 in. (23 x 23cm) . . . . .  
. . . . .P/N 222053

Five small mesh sheets, 4 x 6 in. (10 x 15cm) . . . . .  
. . . . .P/N 222052

## Technical Specifications

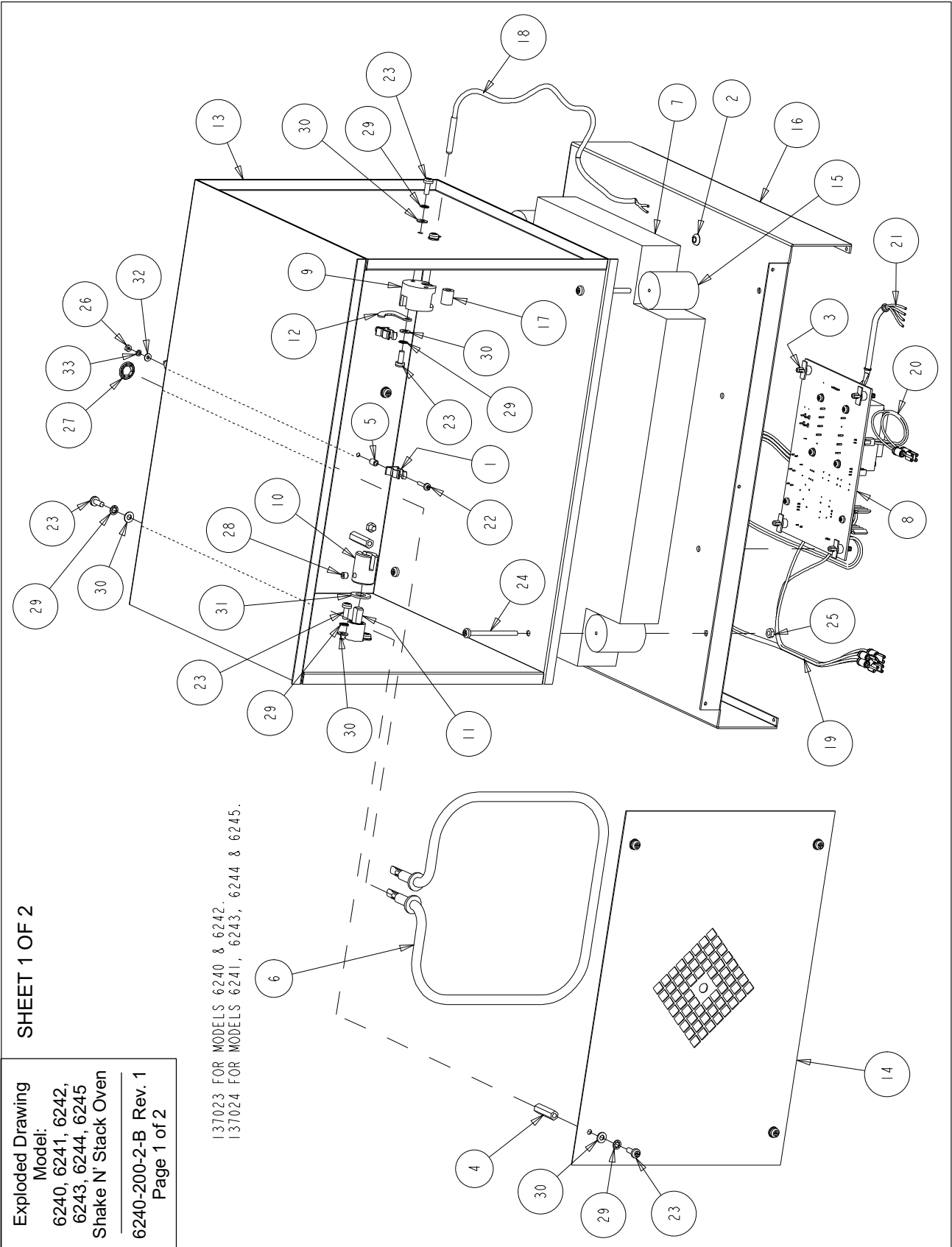
## Technical Specifications



| BILL OF MATERIALS |             |                                    |     |
|-------------------|-------------|------------------------------------|-----|
| ITEM NO.          | PART NO.    | PART DESCRIPTION                   | QTY |
| 1                 | 16041       | #8 INSULATED RING TERMINAL         | 1   |
| 2                 | 102017      | DOOR GASKET FOR SHAKE-N-STACK      | 1   |
| 3                 | 113021      | BLACK ADJUSTABLE FOOT M8X25        | 4   |
| 4                 | 113022      | NON-SLIP SHOE                      | 4   |
| 5                 | 125024      | SPACER                             | 4   |
| 6                 | 140382      | FRONT LABEL                        | 1   |
| 7                 | 140620      | INSULATION BACK                    | 1   |
| 8                 | 140621      | INSULATION LEFT SIDE BOTTOM        | 1   |
| 9                 | 140622      | INSULATION RIGHT SIDE              | 1   |
| 10                | 140623      | INSULATION TOP                     | 1   |
| 11                | 140625      | INSULATION LEFT SIDE TOP           | 1   |
| 12                | 222013-16-1 | OVEN COVER SUB-ASSEMBLY            | 1   |
| 13                | 222013-31-4 | OVEN COVER BACK                    | 1   |
| 14                | 231225      | CONTROLLER GASKET                  | 1   |
| 15                | 231229      | DIGITAL TEMPERATURE CONTROLLER     | 1   |
| 16                | 310186      | PANEL MOUNT POTENTIOMETER          | 1   |
| 17                | 360258      | ROCKER SWITCH WITH GREEN INDICATOR | 1   |
| 18                | 360259      | ROCKER SWITCH WITH BLACK INDICATOR | 1   |
| 19                | 360260      | SPLASH PROOF SWITCH COVER          | 2   |
| 20                | 590043      | M4 X 10mm SS PHP SCREW             | 14  |
| 21                | 610084      | M4 SS HEX NUT                      | 5   |
| 22                | 680021      | M3 X M10 SS PHP SCREW AB POINT     | 7   |
| 23                | 730048      | M4 SS INTERNAL TOOTH LOCK WASHER   | 14  |
| 24                | 730091      | M4 SS FLAT WASHER                  | 17  |

Exploded Drawing  
Model:  
6240, 6241, 6242,  
6243, 6244, 6245  
Shake N' Stack Oven

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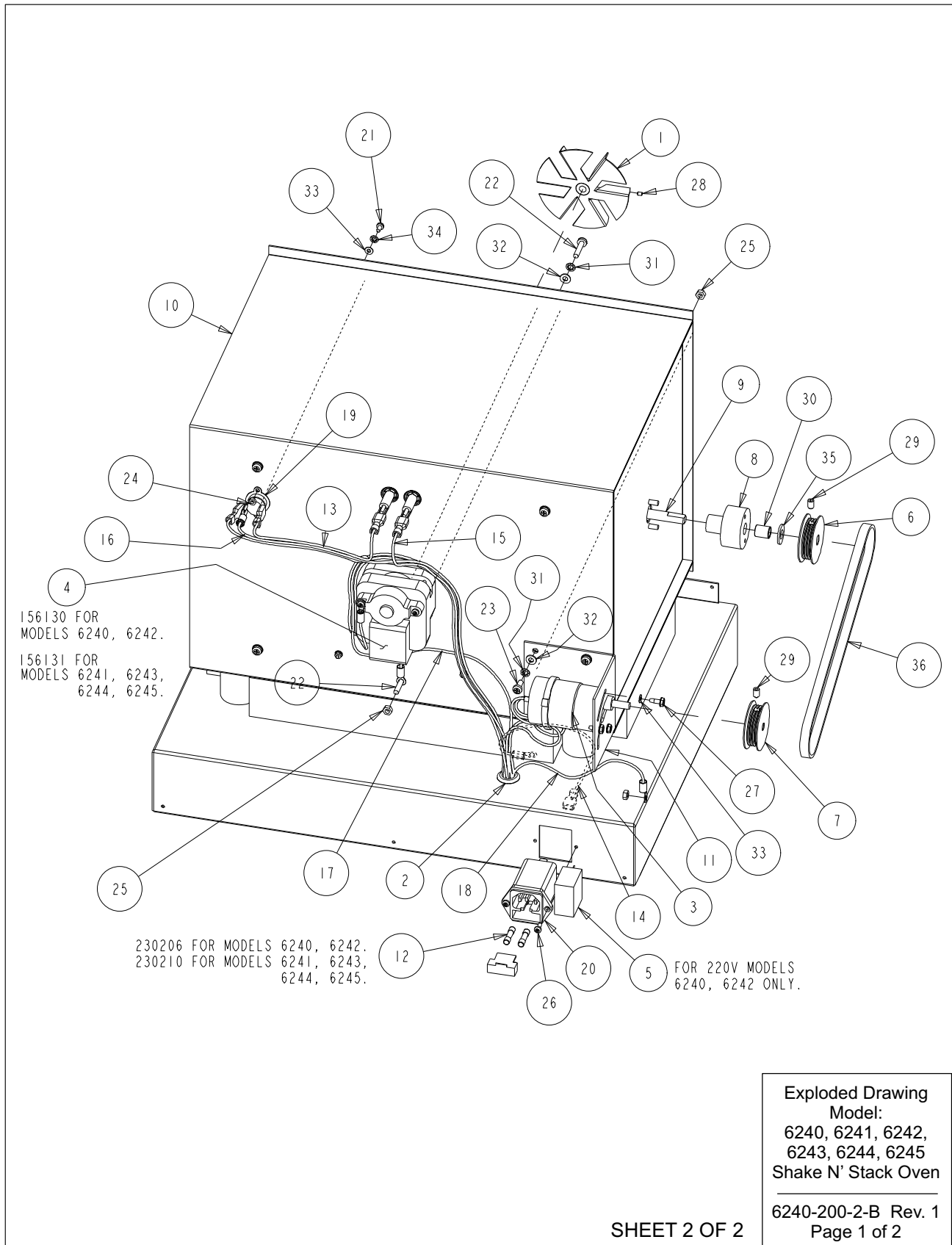


Exploded Drawing  
Model:  
6240, 6241, 6242,  
6243, 6244, 6245  
Shake N' Stack Oven  
6240-200-2-B Rev. 1  
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| BILL OF MATERIALS |             |                                       |     |
|-------------------|-------------|---------------------------------------|-----|
| ITEM NO.          | PART NO.    | PART DESCRIPTION                      | QTY |
| 1                 | 111039      | SPRING CLIP                           | 2   |
| 2                 | 114037      | 6.5MM x 4MM ID GROMMET                | 1   |
| 3                 | 125025      | LOCKING CIRCUIT BOARD SUPPORT         | 4   |
| 4                 | 127082      | M4 X 0.7 BRASS SPACER 25MM LONG       | 4   |
| 5                 | 127083      | 3MM ROUND SPACER 6MM LONG             | 2   |
| 6                 | 137023      | TUBULAR HEATER 240V                   | 1   |
| 7                 | 140624      | INSULATION BOTTOM                     | 1   |
| 8                 | 191770      | MAIN CONTROL PCB ASSEMBLY             | 1   |
| 9                 | 222008      | SHAFT SUPPORT                         | 1   |
| 10                | 222009      | DRIVE COLLAR                          | 1   |
| 11                | 222010      | DRIVE SHAFT                           | 1   |
| 12                | 222011      | LATCH                                 | 1   |
| 13                | 222013-17-1 | OVEN LINER ASSEMBLY                   | 1   |
| 14                | 222013-34-1 | HEATER COVER PLATE                    | 1   |
| 15                | 222016      | OVEN SUPPORT                          | 4   |
| 16                | 222025-31-1 | OVEN LINER SUPPORT                    | 1   |
| 17                | 244024      | 3/16" ID SILICONE TUBING (12mm LONG)  | 1   |
| 18                | 290201      | PTC1000 PROBE SS 6MM X 40MM           | 1   |
| 19                | 350025-3    | MAINS POWER CABLE                     | 1   |
| 20                | 350025-6    | ON/OFF SWITCH CABLE                   | 1   |
| 21                | 350025-7    | TEMPERATURE CONTROL CABLE             | 1   |
| 22                | 590039      | M2.5 X 12mm SS PHP SCREW              | 2   |
| 23                | 590043      | M4 X 10mm SS PHP SCREW                | 17  |
| 24                | 590044      | M4 X 50mm SS PHP SCREW                | 4   |
| 25                | 610084      | M4 SS HEX NUT                         | 10  |
| 26                | 610085      | M2.5 HEX NUT                          | 2   |
| 27                | 650017      | SELF LOCKING RETAINING RING           | 2   |
| 28                | 700059      | M5 X 5MM LONG SS HEX SOCKET SET SCREW | 1   |
| 29                | 730048      | M4 SS INTERNAL TOOTH LOCK WASHER      | 23  |
| 30                | 730091      | M4 SS FLAT WASHER                     | 22  |
| 31                | 730095      | M8 RED FIBER WASHER                   | 2   |
| 32                | 730098      | M2.5 SS FLAT WASHER                   | 2   |
| 33                | 730099      | M2.5 SS SPRING LOCKWASHER             | 2   |

## Section 8

### Technical Specifications

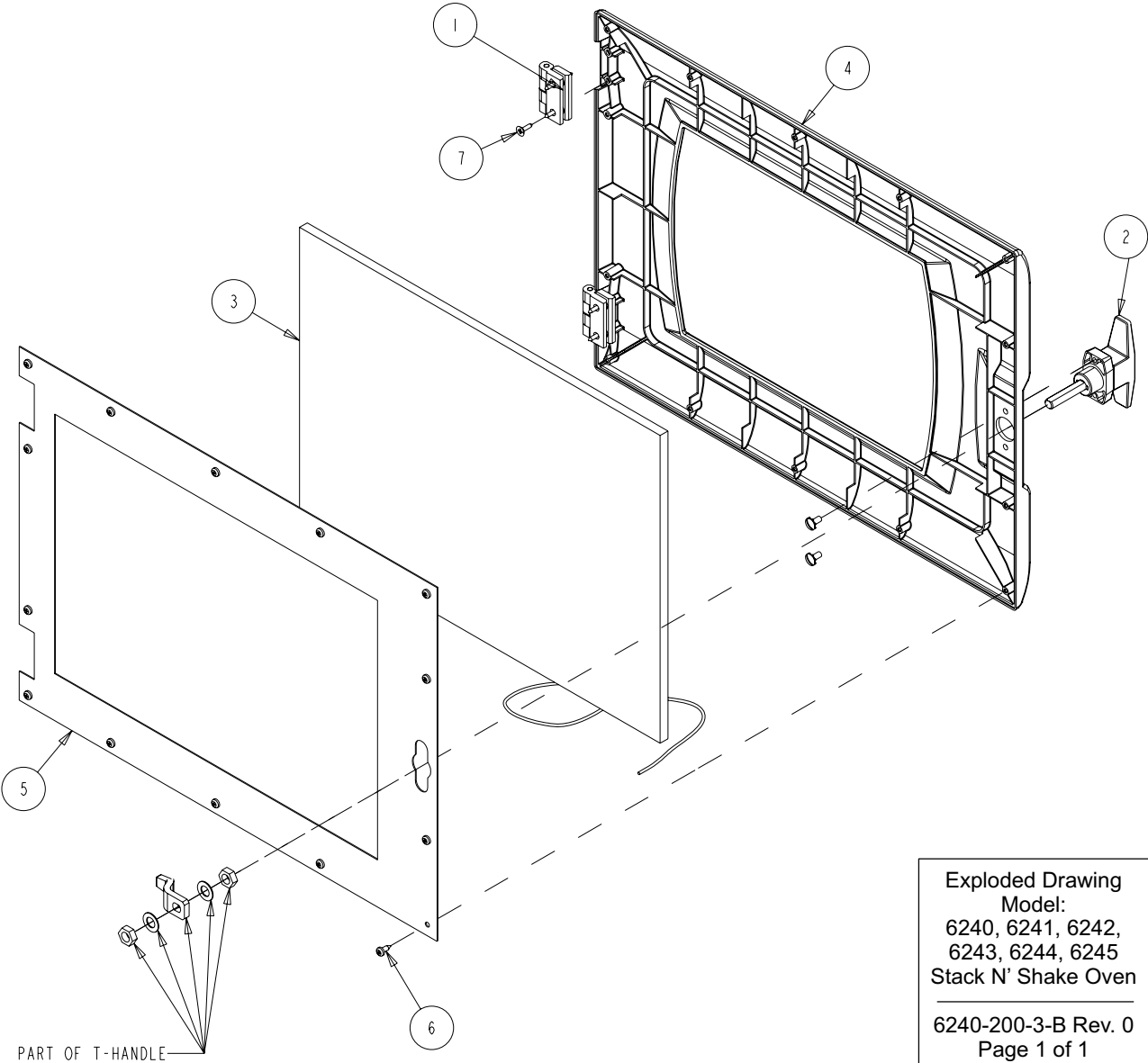


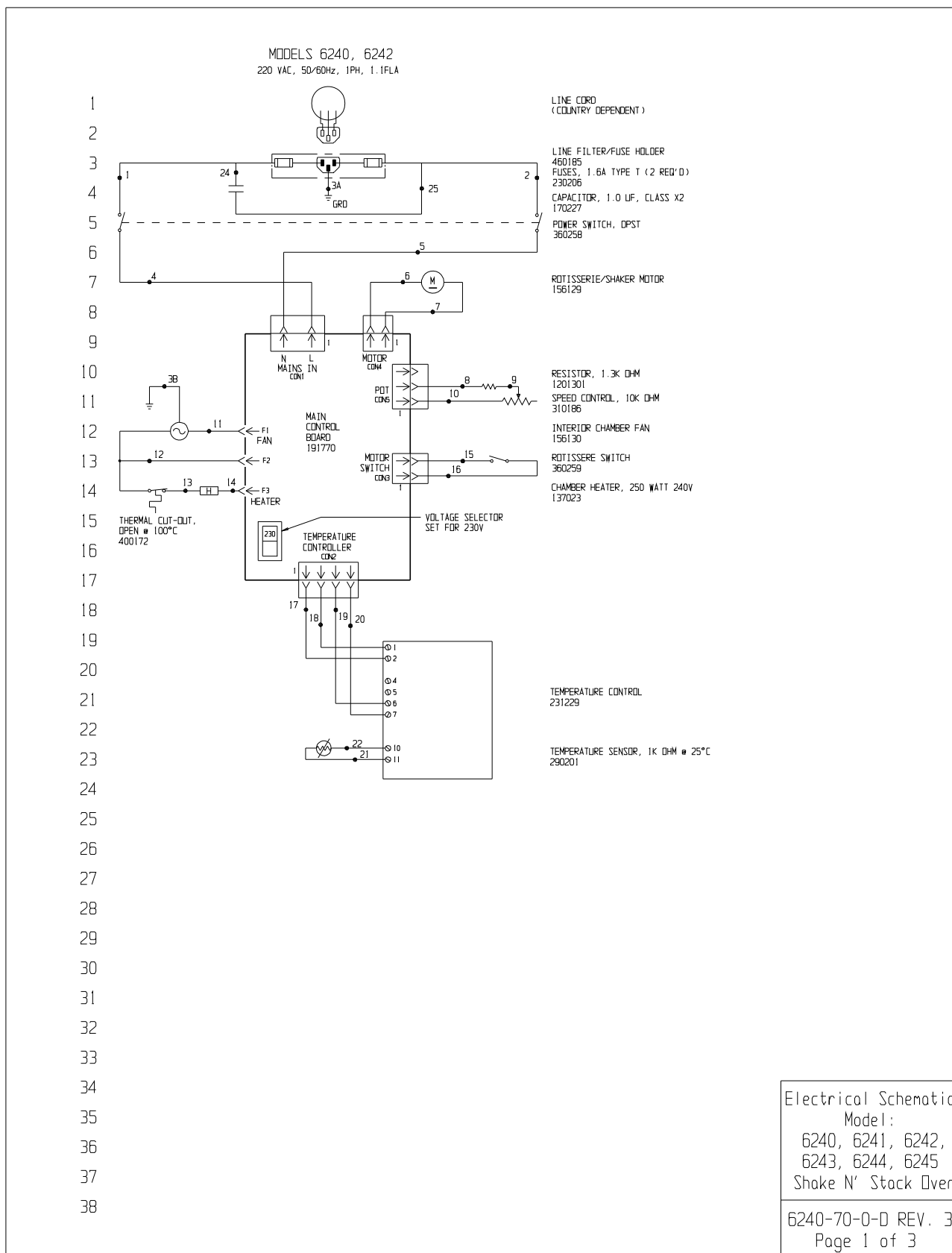
| BILL OF MATERIALS |             |                                                |     |
|-------------------|-------------|------------------------------------------------|-----|
| ITEM NO.          | PART NO.    | PART DESCRIPTION                               | QTY |
| 1                 | 100120      | FAN IMPELLER                                   | 1   |
| 2                 | 114036      | 19MM x 11MM ID GROMMET                         | 1   |
| 3                 | 156129      | 24 VDC TRIDENT GEAR MOTOR                      | 1   |
| 4                 | 156130      | FAN MOTOR - 220V                               | 1   |
| 5                 | 170227      | CAP 1.0UF 275V CLASS X2                        | 1   |
| 6                 | 183021      | IDLER PULLEY                                   | 1   |
| 7                 | 183022      | DRIVE PULLEY                                   | 1   |
| 8                 | 222003      | BUSHING                                        | 1   |
| 9                 | 222010      | DRIVE SHAFT                                    | 1   |
| 10                | 222013-17-1 | OVEN LINER ASSEMBLY                            | 1   |
| 11                | 222013-32-5 | MOTOR BRACKET                                  | 1   |
| 12                | 230206      | FUSE 1.6A 5X20MM                               | 2   |
| 13                | 350025-1    | HEATER TO THERMAL CUTOFF CABLE                 | 1   |
| 14                | 350025-2    | MAINS INLET EARTH GROUND                       | 1   |
| 15                | 350025-4    | PCB TO HEATER CABLE                            | 1   |
| 16                | 350025-5    | PCB TO THERMAL SWITCH CABLE                    | 1   |
| 17                | 350025-8    | COVER EARTH LEAD                               | 1   |
| 18                | 350025-8    | COVER EARTH LEAD                               | 1   |
| 19                | 400172      | TEMPERATURE CUT-OUT                            | 1   |
| 20                | 460185      | FILTERED INLET FUSED                           | 1   |
| 21                | 590036      | M3 X 6mm SS PHP SCREW                          | 2   |
| 22                | 590042      | M4 X 20mm SS PHP SCREW                         | 6   |
| 23                | 590043      | M4 X 10mm SS PHP SCREW                         | 17  |
| 24                | 610083      | M3 SS HEX NUT                                  | 2   |
| 25                | 610084      | M4 SS HEX NUT                                  | 10  |
| 26                | 680021      | M3 X M10 SS PHP SCREW AB POINT                 | 2   |
| 27                | 680024      | #6-32 X 3/8 SELF TAPPING SCREW                 | 3   |
| 28                | 700044      | M3 X 4MM LONG SS HEX SOCKET SET SCREW          | 1   |
| 29                | 700057      | M5 X 8MM LONG ALLOY STEEL HEX SOCKET SET SCREW | 2   |
| 30                | 700058      | SHAFT SUPPORT BEARING                          | 2   |
| 31                | 730048      | M4 SS INTERNAL TOOTH LOCK WASHER               | 23  |
| 32                | 730091      | M4 SS FLAT WASHER                              | 22  |
| 33                | 730092      | M3 SS FLAT WASHER                              | 5   |
| 34                | 730094      | M3 SS INTERNAL TOOTH LOCK WASHER               | 2   |
| 35                | 730095      | M8 RED FIBER WASHER                            | 2   |
| 36                | 800049      | DRIVE BELT                                     | 1   |

Exploded Drawing  
Model:  
6240, 6241, 6242,  
6243, 6244, 6245  
Shake N' Stack Oven

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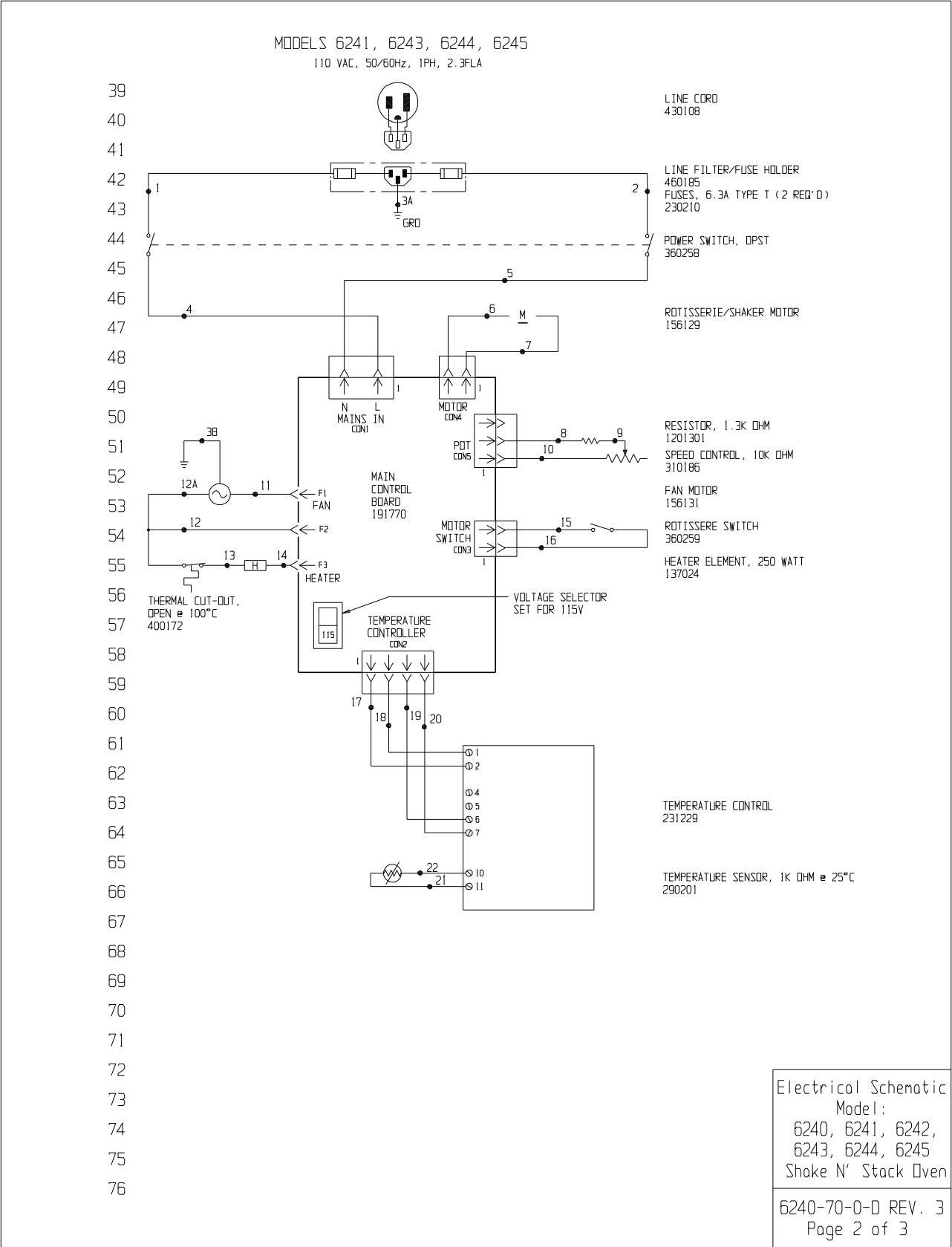
| BILL OF MATERIALS  |             |                                  |     |
|--------------------|-------------|----------------------------------|-----|
| ITEM NO.           | PART NO.    | PART DESCRIPTION                 | QTY |
| 1                  | 116120      | DOOR HINGE                       | 2   |
| 2                  | 121076      | SATIN SILVER T-HANDLE            | 1   |
| 3                  | 179011      | DOOR WINDOW                      | 1   |
| 4                  | 222012      | DOOR MOULDING                    | 1   |
| 5                  | 222013-33-1 | DOOR CLAMP FRAME                 | 1   |
| 6                  | 680020      | M6 x 9.5mm SS PHP SCREW AB POINT | 14  |
| 7                  | 680022      | #8 X 1/2" SS FHP TYPE A SCREW    | 8   |
| BACK_GROUND_WIRING |             |                                  | 1   |





Electrical Schematic  
Model:  
6240, 6241, 6242,  
6243, 6244, 6245  
Shake N' Stack Oven

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Electrical Schematic  
Model:  
6240, 6241, 6242,  
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Shake N' Stack Oven  
6240-70-0-D REV. 3  
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| WIRE REFERENCE CHART |                    |                    |
|----------------------|--------------------|--------------------|
| NO.                  | SIZE               | COLOR              |
| 1                    | .75MM <sup>2</sup> | BROWN              |
| 2                    | .75MM <sup>2</sup> | BLUE               |
| 3A                   | .75MM <sup>2</sup> | GRN/YEL            |
| 3B                   | .75MM <sup>2</sup> | GRN/YEL            |
| 4                    | .75MM <sup>2</sup> | BROWN              |
| 5                    | .75MM <sup>2</sup> | BLUE               |
| 6                    | .5MM <sup>2</sup>  | BLACK              |
| 7                    | .5MM <sup>2</sup>  | RED                |
| 8                    | .5MM <sup>2</sup>  | GREEN              |
| 9                    | N/A                | (RESISTOR LEAD)    |
| 10                   | .5MM <sup>2</sup>  | GREEN              |
| 11                   | .75MM <sup>2</sup> | BLUE (220V MODELS) |
| 11                   | .75MM <sup>2</sup> | RED (110V MODELS)  |
| 12                   | .75MM <sup>2</sup> | BROWN              |
| 12A                  | .75MM <sup>2</sup> | RED                |
| 13                   | .75MM <sup>2</sup> | RED                |
| 14                   | .75MM <sup>2</sup> | PURPLE             |
| 15                   | .22MM <sup>2</sup> | YELLOW             |
| 16                   | .22MM <sup>2</sup> | YELLOW             |
| 17                   | .22MM <sup>2</sup> | BLACK              |
| 18                   | .22MM <sup>2</sup> | BROWN              |
| 19                   | .22MM <sup>2</sup> | RED                |
| 20                   | .22MM <sup>2</sup> | ORANGE             |
| 21                   | SENSOR             | BROWN              |
| 22                   | SENSOR             | BLUE               |
| 24                   | .75MM <sup>2</sup> | BROWN              |
| 25                   | .75MM <sup>2</sup> | BLUE               |

|                                                                |                |          |                |       |     |                |                         |            |                  |
|----------------------------------------------------------------|----------------|----------|----------------|-------|-----|----------------|-------------------------|------------|------------------|
| 3                                                              | DY-368         | 08-13-11 | GSM            | LOC   | CCS | CHG.           | TEMP                    | CONT.      | 231223 TO 231229 |
| 2                                                              | DY-292         | 05-02-08 | JAS            | JAS   | LON | 6240/6242      | COUNTRY                 | DEPENDENT  | CORSET           |
| 1                                                              | DY-181         | 12-18-03 | JAS            | JAS   | LON | CHANGE         | TEMP                    | CONTROLLER | (REL. 2)         |
| 0                                                              | N/A            | 9-25-03  | G <sub>M</sub> |       | MSB | RELEASED       | FOR                     | PRODUCTION |                  |
| REV                                                            | ECN            | NO.      | DATE           | BY    | CAD | APPD           | DESCRIPTION OF REVISION |            |                  |
| MODEL/PART NAME: 6240, 6241, 6242, 6243, 6244 & 6245 SNS OVENS |                |          |                |       |     |                |                         |            |                  |
| DWG TITLE: ELECTRICAL SCHEMATIC                                |                |          |                |       |     |                |                         |            |                  |
| DWN:                                                           | G <sub>M</sub> | CAD:     | G <sub>M</sub> | APPD: | MSB | DATE:          | 8-15-03                 | SCALE:     | NONE             |
| MATERIAL:                                                      |                |          |                |       |     |                |                         |            |                  |
| PAINT COLOR:                                                   |                |          |                |       |     |                |                         |            |                  |
| TOLERANCE UNLESS OTHERWISE SPECIFIED                           |                |          |                |       |     | DRAWING NUMBER |                         | SIZE       |                  |
| ANGLES: DECIMAL: .XX±<br>.XXX±                                 |                |          |                |       |     | 6240-70-0      |                         | D          |                  |



ATTENTION  
OBSERVE PRECAUTIONS  
ELECTROSTATIC  
SENSITIVE DEVICES

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**ThermoFisher**  
**SCIENTIFIC**

BOX 649, MARIETTA, OHIO 45750

Electrical Schematic  
Model:  
6240, 6241, 6242,  
6243, 6244, 6245  
Shake N' Stack Oven

6240-70-0-D REV. 3  
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# Declaration of Conformity

**Manufacturer's Name:** Thermo Fisher Scientific (Asheville) LLC

**Manufacturer's Address:** 401 Millcreek Road  
Marietta, Ohio 45750  
U.S.A.

**Product Description:** Shake and Stack Ovens

**Product Designations:** 6240, 6241, 6242, 6243, 6244, 6245

**Year of Initial CE Marking:** 2005

**Affected Serial Numbers:** Release 3  
(Release Level [REL#] shown on Serial Tag)

This product conforms to the following European Union Directive(s):

**EMC:** 2004/108/EC

**LVD:** 2006/95/EC

This product conforms to the following Harmonized, International and National Standards:

**EMC:**

EN 61326-1  
EN 61000-3-2  
EN 61000-3-3

**LVD:**

EN 61010-1 2<sup>nd</sup> Edition  
UL 61010-1 2<sup>nd</sup> Edition  
CSA C22.2 No. 61010-1 2<sup>nd</sup> Edition

**Date of Declaration August 15<sup>th</sup> 2010**



Eric Pickrell

Regulatory Compliance Specialist

15 August, 2011

**ThermoFisher**  
**SCIENTIFIC**

# Appendix I Troubleshooting Guide for Nucleic Acid Hybridisations

Our bottle system is intrinsically simpler and safer to use than other methods of hybridisation such as hybridisation in bags or plastic boxes. In the Hybridisation Ovens, the temperature of the solutions is precisely controlled and regulated, and, the rotisserie device ensures that the solutions are continuously and evenly distributed over the membrane. Thus, the optimum conditions for hybridisation and washing are maintained throughout when using this system. However, during the transfer of protocols from bags to bottles some minor adjustments to the protocols may be necessary.

When loading the mesh and membranes into the bottles, air bubbles should be avoided. Ensure that the oven is positioned on a flat surface so that the probe solution is distributed evenly along the length of the bottles and that there is sufficient probe solution to cover the entire membrane. On occasion, the mesh and membrane can become tightly rolled up in the bottle. This occurs if the mesh is loaded incorrectly (see Figure 4-1 in Section 4).

## Background Reduction - General

All solutions for nucleic acid hybridisations should be prepared using distilled water and highest quality reagents in clean glassware. In particular, water with a high organic content will cause bad background problems. Formamide should be freshly de-ionised. Membranes should always be handled wearing gloves, or with forceps. The following considerations should also be applied:

### Pre-Hybridisation Procedure

Pre-hybridisation is required to block the sites on the nylon membrane, which the probe would otherwise bind to non-specifically. Failure to carry out adequate pre-hybridisation results in high backgrounds. If dextran sulphate is used in the hybridisation solution, then it must also be included in the pre-hybridisation solution.

## Background Reduction (continued)

### Washing Procedure

Stringency washing should be carried out using large volumes (approximately 100ml) of the following solutions, which should be pre-warmed to the required temperature:

1. 2 x 15 minutes with 2 x SSPE (SSC), 0.1% SDS at 65°C
2. 1 x 30 minutes with 2 x SSPE (SSC), 0.1% SDS at 65°C
3. 1 x 10 minutes with 0.1 x SSPE (SSC), 0.1% SDS at 65°C

All wash solutions should be pre-warmed to the appropriate temperature. An initial room temperature wash is not recommended and can cause background problems.

The final wash is a high stringency wash. Use of a hand-held monitor to give an indication of the decrease in radioactivity as the wash progresses is recommended and should give some indication as to whether this final wash should be carried out.

In general terms, the stringency of hybridisation and washing steps is increased by increasing the temperature, or by decreasing the salt concentration. Hybridisation should be carried out under relatively low stringency conditions compared to the washing procedures. It is generally simpler and more effective to adjust the stringency during the washing steps by altering the salt concentration rather than the temperature.

### Probe Preparation

The final probe concentration should be in the region of 25-50ng/ml of hybridisation solution, at approximately  $1-5 \times 10^6$ cpm/ml.

The optimum length of probe is approximately 500-800bp. Purification of the labelled probe to remove unreacted triphosphates will reduce background problems and is recommended for all hybridisations. Probe solutions should be pre-warmed to the hybridisation temperature and care should be taken to ensure the membrane is not exposed to the concentrated probe solutions if adding it directly to the bottles.

## Factors Resulting in High Backgrounds

1. Hybridisation solutions and/or wash solutions not pre-warmed before use.
2. Probe concentrations too high or probe not denatured. When transferring hybridisation protocols to bottles the volumes will be reduced. Ensure that probe concentrations are adjusted accordingly.
3. Unincorporated nucleotides not removed from probe solution.
4. Insufficient pre-hybridisation or blocking agents in pre-hybridisation and hybridisation solutions (e.g. Denhardt's reagent and salmon sperm DNA). An adequate pre-hybridisation is important to block non-specific hybridisation to the membrane.
5. Hybridisation and/or washing conditions not stringent enough:
  - i) Decrease salt concentration.
  - ii) Increase temperature.
  - iii) Increase concentration of SDS.
  - iv) Increase wash times.
6. Membranes drying out. This may often be the cause of an apparent overlap problem and may result from:
  - i) Too low a probe volume.
  - ii) Too slow a change over of solutions, particularly when bulk processing.
  - iii) Oven not level.
  - iv) Excessive variable axis angle.
7. Residual agarose on membranes may cause foggy backgrounds. Membranes should be rinsed in 2 x SSC to remove residual agarose and excess salt after blotting and prior to fixing (especially following vacuum blotting).
8. Multiple filters not separated by mesh in bottles.
9. Autoradiography problems. Random black spots and "lightening flash" markings on autoradiographs may be due to static electricity.

## Factors Resulting in Signal Lower than Expected

1. Insufficient exposure time of membrane to film during autoradiography.
2. Inefficient transfer and/or binding of nucleic acids to the nylon membrane.
3. Target sequence present at very low copy numbers. Increase the amount of sample loaded on to the gel.
4. Probe sequence not present in sufficient quantities. Increase the concentration of probe or include 10% dextran sulphate, which reduces the solvent volume and has the same effect.
5. No probe homology.
6. Double stranded DNA probe was not denatured - see standard protocols. Alternatively, probe degraded. This is more likely to occur when using RNA probes.
7. The specific activity of the probe was too low. Consider factors such as the probe concentration during the labelling reaction, half-life of radiolabelled triphosphates, etc.
8. Hybridisation and/or washing conditions were too stringent:
  - i) Increase salt concentration.
  - ii) Decrease temperature.
  - iii) Reduce concentration of SDS.
  - iv) Reduce wash times.
9. The hybridisation time was too short.

## Appendix II Hybridisation Bottle Safety

### Use of the Bottle Gripper

- Part No. 222057 - fits 6 in. (15cm) short bottles
- Part No. 222056 - fits 12 in. (30cm) medium, long bottles
- Part No. 222055 - fits 12 in. (30cm) extra long bottles

This silicone rubber safety sleeve fits neatly over all our standard bottles and is included with every oven we ship.

- Helping you to safely tighten and unscrew caps.
- Making handling of hot bottles easier.

For more details and ordering information, contact Technical Services or your local distributor.

### Bottle Care

Our Hybridisation Bottles are made of thick-walled borosilicate glass, which protects users from radiation and has excellent long-term reliability.

- It is important to check your bottles regularly for chips, stress fractures and cracks. If these occur, discard the bottle.
- Ensure bottles are stored either in a suitable rack or with caps replaced between experiments. This will protect the bottle and sealing area.
- Replace O-rings when worn or leaky. Replace all O-rings every six months.
- Wear protective gloves to protect your hands in the event of accidental breakage.
- Never over-tighten caps on bottles. Hand-tight is sufficient.

**Caution** Dropping or over-tightening lids can cause cracking and failure. Lids should be inspected prior to each use. s

- If the bottle cap is difficult to unscrew, NEVER ATTEMPT to force the bottle cap open. Allow the bottle to cool and retry. If the cap remains stuck, discard the bottle.
- The bottles should not be used at temperatures above 70°C.

## THERMO FISHER SCIENTIFIC STANDARD PRODUCT WARRANTY

The Warranty Period starts two weeks from the date your equipment is shipped from our facility. This allows for shipping time so the warranty will go into effect at approximately the same time your equipment is delivered. The warranty protection extends to any subsequent owner during the first year warranty period.

During the first year, component parts proven to be non-conforming in materials or workmanship will be repaired or replaced at Thermo's expense, labor included. Installation and calibration are not covered by this warranty agreement. The Technical Services Department must be contacted for warranty determination and direction prior to performance of any repairs. Expendable items, glass, filters and gaskets are excluded from this warranty.

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Your local Thermo Sales Office is ready to help with comprehensive site preparation information before your equipment arrives. Printed instruction manuals carefully detail equipment installation, operation and preventive maintenance.

If equipment service is required, please call your Technical Services Department at 1-800-438-4851 (USA and Canada) or 1-740-373-4763. We're ready to answer your questions on equipment warranty, operation, maintenance, service and special application. Outside the USA, contact your local distributor for warranty information.



Rev. 4 4/09

## **THERMO FISHER SCIENTIFIC INTERNATIONAL DEALER WARRANTY**

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Your local Thermo Sales Office is ready to help with comprehensive site preparation information before your equipment arrives. Printed instruction manuals carefully detail equipment installation, operation and preventive maintenance.

Contact your local distributor for warranty information. We're ready to answer your questions on equipment warranty, operation, maintenance, service and special application.



Rev. 4 2/09



# **HYBRIDISATION GUIDE**

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## **USER INSTRUCTION MANUAL**

**Manual 7222060 Rev. 0**



# HYBRIDISATION GUIDE

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**HYBRIDISATION BOTTLE SAFETY:** It is important to follow correct safety procedures when using Hybridisation Bottles. Please see Appendix II for details

# CHAPTER 1

## HYBRIDISATION GUIDE

### INTRODUCTION

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The immobilisation of nucleic acids on a solid support and subsequent detection of specific sequences of interest is one of the fundamental techniques of modern molecular biology. A number of different techniques may be used to analyse target sequences from various sources. These techniques are all variations on a theme, which can be summarised according to the source of DNA or RNA as follows: -

1. **Dot/Slot Blot Hybridisation**

A sample of unfractionated DNA or RNA immobilised on a membrane.

2. **Colony/Plaque Blot Hybridisation**

DNA derived from cultured bacteria directly or from bacteriophage plaques and immobilised on a membrane.

3. **Southern/Northern Blot Hybridisation**

DNA or RNA, which has been size fractionated by gel electrophoresis and then transferred to a membrane.

4. **Western Blot Hybridisation**

Western blotting refers to the detection of polypeptides blotted on to nitrocellulose with antibodies, and is outside the scope of this manual.

In each case, the basic principle remains the same. The nucleic acid for analysis is immobilised on a solid support and then hybridised with a specific, single-stranded, Hybridisation 'probe' complementary in base sequence to the region of interest, and labelled to facilitate its detection.

The solid support most widely used for Hybridisation reactions is the nylon Hybridisation membrane. This offers several advantages over alternative matrices such as nitrocellulose, most notably high physical strength and binding capacity. The DNA is immobilised on the support by baking or UV cross-linking.

The Thermo range of Hybridisation equipment offers a complete system for the safest and most efficient processing of all types of blot. The equipment is invaluable for every stage of the nucleic acid immobilisation and detection procedure, from the initial electrophoretic separation, through to vacuum transfer and finally, Hybridisation and washing. The procedures outlined in this booklet are intended as general guidelines for the use of Thermo Hybridisation Ovens and may not be ideally suited for every research application.

For further information, the user is recommended to refer to the following laboratory manuals (in addition to literature cited in the references): -

**Fritsch, J. & Maniatis, T. (1989)**

Molecular Cloning: A Laboratory Manual

*2nd Edition Sambrook, Cold Spring Harbour Laboratory Press*

**Edited by: Hames, B.D. & Higgins, S.J. (1985)**

Nucleic Acid Hybridisation: A Practical Approach

*IRL Press, Oxford*

## CHAPTER 2

### HYBRIDISATION GUIDE

### NUCLEIC ACID TRANSFER PROCEDURES

---

#### ***Dot/Slot Blotting***

1. DNA samples, prepared from cell lysate, must first be denatured to form single-stranded DNA. Heat to 100°C and incubate for 5 minutes.
2. Chill DNA samples on ice for 5 minutes.
3. Cut two pieces of Whatman 3MM™ filter paper and one sheet of membrane to the size of the Dot blotting unit. Pre-wet the membrane and filter paper in 6 x SSPE (SSC), then place in the unit and secure the lid. If samples are to be spotted on manually, pre-wet the membrane in 6 x SSPE (SSC) and place on dry filter paper.
4. Pipette samples into the wells for binding of the DNA to the membrane and follow manufacturer's instructions to operate apparatus correctly.  
  
If samples are spotted manually, apply 0.5-1.0µl aliquots and allow to dry between applications to prevent excessive spreading.
5. Dismantle the apparatus and remove the membrane.
6. Place the membrane on a sheet of Whatman 3MM™ filter paper saturated with **denaturing solution** for 1 minute.
7. Transfer the membrane to a sheet of filter paper saturated with **neutralising solution** for one minute.
8. Place the membrane on dry filter paper and allow to air dry.
9. Fix the DNA by baking the membrane for 1 hour at 80°C, or by UV cross-linking. UV cross-linking is the most efficient method of fixing the DNA to Hybridisation membranes and is particularly useful if reprobing is to be carried out.
10. After fixing the DNA the membrane is ready for Hybridisation. Membranes not used immediately may be stored between sheets of Whatman 3MM™ in sealed plastic bags at 4°C.

## Colony/Plaque Blotting

### *Colony Blotting*

1. Grow bacteria on agar containing the appropriate selective antibiotic until colonies are approximately 0.5-1mm in diameter.
2. Place a Hybridisation membrane cut to the size of the plate on to the surface of the agar. Orientation points should be marked at this stage by piercing the membrane at three asymmetric points with a sterile needle.
3. Replica filters may be prepared from the initial colony lift at this stage by pressing a second Hybridisation membrane firmly on to the template membrane for a few seconds. The simplest way to do this is by sandwiching the membranes between glass plates lined with a few sheets of Whatman 3MM™ paper, and pressing them firmly together. Carefully separate the membranes to avoid smearing the colonies. This replica, and any subsequent replicas, should also be marked with orientation points.
4. The replica membranes are then placed colony side up on to fresh agar plates containing the appropriate selective antibiotic and incubated at 37°C until colonies of 0.5-1mm are present on the surface of the membrane. At this stage, it is possible to amplify low copy number plasmids (relative to cellular DNA) by transferring the filters on to agar plates containing chloramphenicol at 200µg/ml and incubating for 10 hours at 37°C.
5. Place the Hybridisation membranes on Whatman 3MM™ paper saturated in **neutralising solution** for 3 minutes then repeat with fresh **neutralising solution**.
6. Rinse the membranes briefly in 2 x SSPE (SSC), then air-dry on a sheet of filter paper.
7. Fix the DNA to the membranes either by baking for two hours at 80°C, or by UV crosslinking.
8. After fixing the DNA, membranes may be stored between sheets of Whatman 3MM™ paper in sealed plastic bags at 4°C.
9. Prior to Hybridisation, it may be advantageous to carry out a pre-washing step to remove colony debris and hence reduce background noise.

Pre-washing is carried out as required by incubating in at least 200ml of **prewash solution** (see Appendix I) for at least 30 minutes at 50°C. Longer incubations and several changes of buffer will assist in pre-washing. When the colonies are sufficiently broken up, residual colony debris may be removed by gently rubbing the colonies with a gloved finger.

This procedure is particularly advantageous when screening with oligonucleotides.

### ***Plaque Blotting***

The above procedure, with some minor modifications may also be utilised for the screening of bacteriophage plaques as follows: -

1. Aliquots of the bacteriophage stock should be mixed with plating cells and plated in soft agarose. Incubate at 37°C until plaques are approximately 0.2-0.5mm in diameter (approx. 10-12 hours).
2. Chill the plates for about 1 hour to set the top agarose.
3. Place a Hybridisation membrane cut to size (or pre-cut disc) on to the surface of the agarose and leave for at least 30 seconds. Orientation points should be marked with a sterile needle. Further replicas may be prepared by leaving the Hybridisation membrane for progressively longer periods of time on the surface of the agarose.
4. To process membranes further; proceed as from stage number 5 as for 'Colony Blotting'. Pre-washing the filters (stage 9) should not be necessary.

### ***Southern Blotting***

1. Size fractionation of the DNA is carried out by agarose gel electrophoresis (a suitable range of horizontal gel apparatus is available from Thermo Scientific). Before transfer to Hybridisation membranes, the DNA in the agarose must be treated to ensure efficient transfer and to generate single-stranded DNA suitable for Hybridisation. Gentle agitation of the gel is essential to prevent damage to the gel during these steps. The shakers in Shake 'n' Stack, Midi Dual 14 and Maxi 14 are ideal for this purpose.
2. Depurination to break the DNA into smaller fragments suitable for transfer is recommended for transfer of all DNA fragments large than 10kb and may assist transfer of smaller fragments. Place the gel in a solution of 0.25M HCl for 10 minutes at room temperature with gentle shaking.

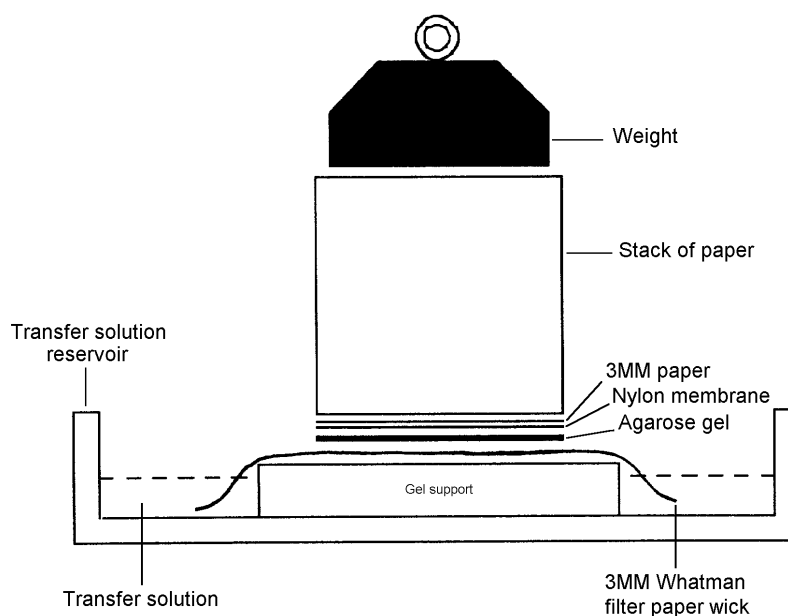
3. Replace the **depurinating solution** with **denaturing solution**. Denaturation for at least 30 minutes is recommended for most applications with a change of solution after 15 minutes.
4. Replace **denaturing solution** with **neutralising solution** and gently shake the gel for least 30 minutes, replacing with fresh solution after 15 minutes.
5. The DNA is then transferred to a Hybridisation membrane cut to the size of the gel. At this stage, a corner of the membrane should be cut to assist later in orientation. Transfer may be carried out by capillary blotting, or more efficiently by vacuum blotting. Vacuum blotting offers significant advantages over capillary blotting, namely: -
  - a) Transfer is completed in 1 hour, instead of at least 4 hours for capillary blotting, or more usually overnight.
  - b) Superior results are obtained with vacuum blotting. Because of the rapid transfer time, there is less lateral diffusion of the DNA during transfer to the Hybridisation membrane. This results in sharper bands on autoradiographs. Excellent blots may be prepared, quickly and conveniently, using the Thermo Vacu-Aid apparatus.

### **Capillary Blotting**

1. Capillary blotting requires a buffer reservoir containing 10-20 x SSPE (SSC) (e.g. a bioassay dish) with a raised support for the gel (e.g. an inverted sandwich box). Two sheets of Whatman 3MM™ filter paper cut to the same width and slightly longer than the gel should be pre-wet in 10-20 SSPE (SSC) and placed across the raised support so that the ends are submerged in the buffer reservoir. This serves as a wick for the flow of the buffer through the gel (see *Figure 2.2: Capillary Blot Assembly*).
2. Invert the gel and place centrally on the filter paper. Any filter paper exposed at the edge of the gel should be covered in Saran Wrap™, used X-ray film or similar, to prevent flow of buffer around the edge of the gel.

3. Place a sheet of Hybridisation membrane cut to the size of the gel and marked for orientation on to the surface of the gel, taking care to avoid air bubbles. The membrane should only be handled wearing gloves or using forceps. Then cover the membrane with two sheets of Whatman 3MM™ paper cut to just larger than the gel.
4. Place a stack of absorbent paper towels on top of the filter paper, weighted down with a 1kg weight. The stack of towels should be approximately 5cm deep, sufficient to continue drawing buffer through the gel until transfer is complete, which takes at least 4 hours. The stack of towels should also be level to ensure even transfer. Take care to ensure that the stack of towels is not in contact with the buffer wick, which would cause a short circuit of buffer, bypassing the gel. Usually it is convenient to transfer overnight.
5. After blotting is complete, the apparatus is dismantled. At this stage it is possible to mark the position of the wells on the Hybridisation membrane with a soft pencil. The membrane is then rinsed briefly in 2 x SSPE (SSC) and air-dried on a sheet of dry filter paper.
6. Fix the DNA to the Hybridisation membrane, either by baking at 80°C for 2 hours, or by UV cross-linking.
7. After fixing the DNA, the membrane is ready for Hybridisation. Membranes not hybridised immediately may be stored between sheets of Whatman 3MM™ paper in sealed plastic bags at 4°C.

**Figure 2.1:** Capillary Blot Assembly



## Northern Blotting

Electrophoresis of RNA should be carried out in a denaturing gel system. Maniatis *et al* 1989 gives details of running denaturing RNA gels using formaldehyde or glyoxal.

1. After electrophoresis of the RNA in a denaturing gel the transfer can be set up as described for DNA gels (see Southern Blotting above). The denaturing step 3 and the neutralising step 4 of the gel are not required, as the RNA is denatured in the gel. However, some researchers recommend reduced stringency denaturation and neutralisation steps to facilitate transfer of large RNA molecules as follows: -

|                      |            |
|----------------------|------------|
| 50mM NaOH, 10mM NaCl | 30 minutes |
| 100mM Tris HCl Ph7.5 | 30 minutes |

Gentle agitation of the gel is essential to prevent damage to the gel during these steps. The shaker in the Shake 'n' Stack, Midi Dual 14 and Maxi 14 are ideal for this purpose.

2. RNA transfer is carried out in 10-20 x SSPE, using the same procedure as outlined for DNA gels in Southern Blotting section above.
3. After transfer of the RNA to the Hybridisation membrane is completed, fixing of the RNA is carried out by baking at 80°C for 2 hours, or by UV cross-linking.
4. After fixing the RNA, the membrane is ready for Hybridisation. Membranes not used immediately may be stored between sheets of Whatman 3MM™ paper in sealed plastic bags at 4°C.

## CHAPTER 3

### HYBRIDISATION GUIDE

### HYBRIDISATION PROCEDURES

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Nucleic acid hybridisations are conveniently performed in the dedicated Hybridisation equipment available from Thermo.

The Hybridisation Oven range consists of incubators with integral rotisserie devices, which can accommodate 4, 10 or 14 Hybridisation bottles, 35mm in diameter, in order to perform Hybridisations in minimal probe volumes with fluid moving continually over the membrane.

#### ***Southern Blot DNA Hybridisations***

The following protocol is broadly applicable to Hybridisations using DNA probes of 50bp and above, following transfer and fixing of DNA to membranes as described in Chapter 2. For Hybridisations using oligonucleotides refer to Chapter 6. The Hybridisation procedure consists of four stages: -

1. Prehybridisation
2. Hybridisation
3. Stringency washing
4. Autoradiography

For detailed notes on Hybridisation specific to Thermo equipment, refer to 'Notes for Nucleic Acid Hybridisation' on page 11.

1. **Prehybridisation** is carried out by incubating the membrane in **Southern Blot Prehybridisation Buffer** (for recipe see page 30). Denature salmon sperm DNA by boiling for 5 minutes and then chilling on ice. Add the denatured salmon sperm DNA to the buffer to a final concentration of 50µg/ml.
2. Incubate with agitation or in a rotisserie for a least 1-hour at 65°C.
3. The volume of prehybridisation buffer required varies according to the Hybridisation system being utilised. In general terms, the minimum volume of buffer should be used such that the membrane is covered by the fluid at all times (approximately 0.1ml/cm<sup>2</sup>), or if in Hybridisation bottles, 10ml for a large bottle and 5ml for a small bottle.

4. Denature the purified labelled probe by heating to 100°C and incubating for 5 minutes, then chill on ice and either add to the prehybridisation solution taking care not to pipette directly on to the membrane, or dilute the probe in pre-washed Hybridisation solution and use this to replace the prehybridisation solution.
5. **Hybridise** with agitation, or by rotating in bottles, for approximately 12 hours at 65°C.
6. Stringency washing steps are carried out as follows, using large volumes (at least 50ml) of the following solutions, which should be pre-warmed to the required temperature: -

|                |                       |                  |
|----------------|-----------------------|------------------|
| 2 x 15 minutes | with 2 x SSPE (SSC)   | 0.1% SDS at 65°C |
| 1 x 30 minutes | with 1 x SSPE (SSC)   | 0.1% SDS at 65°C |
| 1 x 10 minutes | with 0.1 x SSPE (SSC) | 0.1% SDS at 65°C |

The final wash is a high stringency wash. Use of a hand held monitor to give an indication of the decrease in radioactivity as the washes progress is recommended and should determine whether this final wash should be carried out. (See Appendix II for further notes.)

7. Wrap the membrane in Saran Wrap™ and autoradiograph at -70°C in a cassette with an intensifying screen or use a phosphorimager. Expose initially for approximately 12 hours (or overnight).
8. If the membrane is to be reprobbed, at no stage should the membrane be allowed to dry out? Removal of the probe on the membrane may be carried out by boiling the membrane in 0.1 x SSPE (SSC), 0.1% SDS for 5 minutes. Check for residual probe activity by autoradiography before hybridising again.

## ***Northern Blot RNA Hybridisation***

1. Prehybridisation is carried out by incubating the membrane in **Northern Blot Prehybridisation Buffer** (recipe on page 33). Denature salmon sperm DNA by boiling for 5 minutes and then chilling on ice. Add the denatured salmon sperm DNA to the buffer, to a final concentration of 100µg/ml.
2. Incubate with agitation or in a rotisserie for at least one hour at 42°C.
3. The volume of prehybridisation buffer required varies according to the Hybridisation system being utilised. In general terms, the minimum volume of buffer should be used such that the membrane is covered by the fluid at all times (approximately 0.1ml/cm<sup>2</sup>), or if in a Hybridisation bottle, 10-20ml for a large bottle and 5-10ml for a small bottle.
4. Denature the labelled probe by heating to 100°C and incubating for 5 minutes. Chill on ice and add to the prehybridisation solution. Depending upon the system utilised, the probe may be added directly or, alternatively, some prehybridisation buffer is removed, the probe added to this, and then the solution replaced in the Hybridisation vessel. Some researchers may prefer to use fresh Hybridisation solution.
5. Hybridise with agitation, or rotating in bottles, for approximately 12 hours at 42°C.
6. Stringency washing steps are carried out as follows using large volumes (at least 50ml) of the following solutions which should be pre-warmed to the required temperature: -

|                |                 |                  |
|----------------|-----------------|------------------|
| 2 x 15 minutes | with 2 x SSPE   | 0.1% SDS at 42°C |
| 1 x 30 minutes | with 1 x SSPE   | 0.1% SDS at 42°C |
| 1 x 15 minutes | with 0.1 x SSPE | 0.1% SDS at 42°C |

The final wash is a high stringency wash. Use of a hand held monitor to give an indication of the decrease in radioactivity as the washes progress is recommended and will determine if the final wash should be carried out.

7. Wrap the membrane in Saran Wrap™ and autoradiograph at -70°C in a cassette with an intensifying screen or use a phosphoimager. Expose initially for 12 hours (or overnight).
8. If the membrane is to be reprobbed, at no stage should the membrane be allowed to dry out? Removal of the probe on the membrane may be carried out by washing the membrane for 1-2 hours at 65°C in 5mM Tris HCl pH8.0, 0.2mM EDTA, 0.1 x Denhardt's reagent.

### ***Notes for Nucleic Acid Hybridisations using the Thermo Range of Equipment***

The Hybridisation Oven range has been designed to provide the optimum conditions for performing all types of Hybridisation and stringency washing procedures safely and simply. Hybridisations are performed in bottles to maximise user safety and to minimise probe volumes.

#### ***Placing Membranes in a Bottle***

1. Place the bottles to be used in the Oven during the warm up period (approximately 1 hour).
2. Select a piece of support mesh appropriate for the size of the membrane. The recommended Hybridisation mesh is supplied by Thermo.
3. Pre-wet the mesh and Hybridisation membrane in a suitable tray containing 2 x SSPE (SSC) (see *Figure 3.1*).
4. Ensuring that the Hybridisation membrane exactly overlays the mesh, roll both up into a tight roll.

If more than one membrane is to be hybridised in a bottle, simply overlay further meshes and membranes as required before rolling. It is important that each membrane is separated from any other by a piece of mesh. Up to five 20 x 20mm membranes can be hybridised in a single Hybridisation bottle.

5. Place 10-15mls SSPE (SSC) into a Hybridisation Bottle and then insert the roll in such a way that the leading edge (inside the roll) and the trailing edge are positioned relative to each other as shown in Figure 3.2).
6. Place the bottle on a flat surface and then slowly unwind the membrane and mesh around the inside of the bottle by rocking and gently rolling the bottle along the surface. No air bubbles should be visible between the membrane and the bottle. If bubbles are present, the membrane should be removed and re-rolled.

The procedure should then be repeated more gently. Rock the bottle backwards and forwards to attach the first part of the membrane to the bottle. Then roll the bottle in order to unwind the mesh and membrane.

7. Continue until the membrane and mesh is fully unwound. The mesh ensures the probe will have access to all parts of the Hybridisation membrane including those parts of the membrane, which overlap.

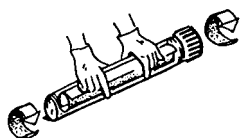
**Figure 3: Inserting Membranes in a Bottle**



1. Shallow tray containing 2 x SSPE (SSC).  
Mesh.  
Membrane.  
Roll up mesh & membrane.



2. Place 10-15mls of SSPE (SSC) into a bottle and then insert the roll centrally.



3. Secure cap and, holding bottle horizontally, roll to catch the trailing edge of the mesh, continue rolling in the same direction until coil of mesh and membrane is well positioned.



4. Pour out SSPE (SSC) and replace with pre-warmed prehybridisation buffer.
5. Place the bottle in the Oven so that it rotates in the Oven in the same direction, as it was unrolled in step 3.

## ***Prehybridisation***

1. Once the membrane is in place in the bottle the SSPE (SSC) can be discarded and replaced with prehybridisation buffer.

This is easily done by removing the cap, pouring off the SSPE (SSC) and then pouring in the prehybridisation buffer. All the solutions used during Hybridisation should be pre-warmed before use. 10-20ml solution is recommended for medium bottles and 5-10ml for small bottles. Higher volumes will be required if there is more than one membrane in the bottle, e.g. 15-25ml.

2. Replace the cap on the bottle and insert into the rotisserie.

## ***Hybridisation***

1. Denature the purified probe by boiling for 5 minutes, then store on ice. If the volume to be used for Hybridisation is substantially different to that used previously for Hybridisation in bags or boxes, ensure that the quantity of the probe is adjusted accordingly to maintain the correct probe concentration. If this is not done, high background may result.
2. Remove the bottle from the Hybridisation Oven and unscrew the cap.
3. If the same buffer is to be used for Hybridisation as for prehybridisation simply pipette the probe into the prehybridisation fluid in the bottle. Take care to avoid pipetting the probe directly on to the membrane as this will result in hot spots. Alternatively, dilute the probe in pre-warmed Hybridisation buffer outside the bottle. Pour off the prehybridisation buffer and replace with the probe solution.
4. Replace the cap and gently agitate the bottle to ensure an even distribution of the probe in the Hybridisation solution.
5. Place the bottle back in the Hybridisation Oven, switch on the rotisserie and leave it to hybridise for the required time period.

## ***Washing***

### ***Method 1 - Washing in Hybridisation Bottles***

1. Remove the bottle from the Oven.
2. Pour off the Hybridisation buffer and then **half fill** the bottle with the first pre-warmed wash solution (approx. 100ml) and replace the cap.

**NOTE: Room temperature washing or washing with solutions, which have not been pre-warmed, may result in background problems (see Chapter 7, page 27).**

3. Replace the bottle in the Oven and leave for the required time period.

If transferring from sandwich box or plastic bag methodology, the times and temperatures are usually those, which are currently being used, approximately 20 minutes per wash step.

4. Repeat steps 1-3 for each additional wash. The wash solutions, temperatures etc., should be those recommended by the membrane manufacturer, or as detailed in Chapter 7, Washing Procedure.

**NOTE: All wash solutions should be pre-warmed for best results.**

### ***Method 2 - Washing in a Sandwich Box***

Some scientists prefer to remove the membranes from the bottles and wash them all in one container. Washing may be performed very simply on the shaking platform in the Shake 'n' Stack, Midi Dual or Maxi 14 Ovens and is particularly useful when several membranes are being processed simultaneously.

1. Remove the Hybridisation Bottle from the Oven. Pour off the Hybridisation buffer.
2. Using a pair of forceps, carefully remove the membranes and place them in a suitable container with a lid (e.g. sandwich box or similar).
3. Using the forceps gently unroll the membranes.

4. Add a sufficient volume of the pre-warmed first wash solution to totally immerse the membranes. Replace the lid and shake on the shaking platform in the Hybridisation Oven at the required temperature for the specified time period.

**NOTE: Initial room temperature washes are not recommended and may result in subsequent background problems.**

5. Remove the first wash solution and replace it with an equal volume of the pre-warmed second wash solution. Replace the lid and shake at the required temperature for the required time period.
6. Step 5 should be repeated for any subsequent wash. The washing protocol using the shaking platform will be identical to that used in the bottles.

*Please refer to Chapter 7 for additional information on Washing.*

## CHAPTER 4

### HYBRIDISATION GUIDE

### NUCLEIC ACID PROBES

---

There are now several types of nucleic acid probe available to researchers and a wide variety of radio-labelling techniques for labelling these probes.

For convenience, the types of nucleic acid probe may be classified as follows: -

1. Double-stranded DNA probes
2. Single-stranded DNA probes
3. RNA probes
4. Synthetic oligonucleotides

#### ***Double-Stranded DNA Probes***

Any suitable DNA molecules (cloned or uncloned) can be used as a Hybridisation probe, for example, insert fragments from a DNA library may be excised from plasmids or bacteriophages by restriction enzyme digest and then labelled for Hybridisation. Another possibility is to use the Polymerase Chain Reaction to synthesize copies of the region of interest, which may be radio-labelled during the amplification reaction, itself, or the final product labelled post-amplification. Labelling of double-stranded DNA may be carried out by NICK TRANSLATION OR PRIMER EXTENSION. Commercially available kits enable these techniques to be carried out simply and efficiently resulting in probes of high specific activity, often requiring no further purification before adding to the Hybridisation buffer.

Removal of unincorporated nucleotides may be advantageous in reducing background. This may be simply carried out using Thermo Recovery kits.

#### ***Single-Stranded DNA Probes***

Single-stranded DNA probes may be synthesized from mRNA using Reverse Transcriptase, or may be derived from fragments cloned into specialised M13 or phagemid vectors, which contain the origin of replication of a single-stranded DNA bacteriophage. Synthesis of the DNA strand complementary to the region of interest incorporating a  $^{32}\text{P}$  labelled dNTP results in a single-stranded radio labelled probe molecule, which is then separated from the unlabelled template by gel electrophoresis (the fragment may then be extracted from the gel using Thermo Recovery kits). This technique eliminates the possibility of re-association of complementary strands, which can occur with double-stranded DNA probes.

## **RNA Probes**

RNA probes are also derived from fragments, which have been cloned into specialised cloning vectors. These vectors contain the promoter sequences of a bacteriophage encoded DNA - dependent RNA polymerase (for example SP6 to T7) adjacent to a multiple cloning site, into which the fragment is inserted. Transcription of the cloned fragment in the presence of the specific RNA polymerase and a  $^{32}\text{P}$ -labelled rNTP results in labelled RNA. The template DNA is removed simply by DNase digest. Vectors of this type are available containing two phage promoters in opposite orientations adjacent to the multiple cloning site so that the strand to be transcribed may be chosen according to the phage RNA polymerase used. These RNA probes have become the probes of choice for many applications, the only limitations being the additional care required for handling RNA.

## **Synthetic Oligonucleotides**

An oligonucleotide probe of a single defined DNA sequence may be synthesized if the target nucleic acid sequence is available using a DNA synthesis machine or commercial service. Alternatively, pools of oligonucleotides of degenerate sequences may be synthesized, derived from an amino acid sequence of a gene product. Due to the degeneracy of the genetic code (i.e. most amino acids are specified by more than one triplet codon) a given amino acid sequence will be coded for by a number of alternative DNA sequences, one of which will represent the specific target sequence for the probe. Oligonucleotides are most commonly radio-labelled using T4 Polynucleotide Kinase to add a labelled phosphate group to the 5' terminus.

The various protocols of radio-labelling nucleic acid probes are widely available in the published literature (e.g. Maniatis *et al*). In addition, there are several commercially available kits containing all the components required for the different types of labelling experiments.

High quality HPLC purified oligonucleotides are available online from Thermo Scientific, visit our web site at [www.thermo.com/molecularbiology](http://www.thermo.com/molecularbiology).

## CHAPTER 5

### HYBRIDISATION GUIDE

#### NON-RADIOACTIVE SYSTEM

---

The use of non-radioactive labelling techniques is becoming increasingly important for nucleic acid Hybridisation. There are currently a number of systems available. Most are based on the labelling of probes with either biotin or the hapten Digoxigenin (DIG).

Biotinylated probes are hybridised and then linked to an appropriate enzyme such as alkaline phosphatase. Visualisation is achieved by reaction of the enzyme with a chromogenic substrate.

Similarly, DIG labelled probes are detected by high affinity Fab fragments coupled to alkaline phosphatase. Visualisation is achieved using chromogenic substrates such as NBT and BCIP, which produces a dark blue colour.

Although both of these systems offer advantages over radio-labelled probes (no safety risk or stability problems), they have been recognised as less sensitive.

Recently the sensitivity of detection has been increased significantly (down to femtogram levels) by using a chemiluminescent reaction. The Böehringer DIG system with the same alkaline phosphatase conjugate will generate light with the chemiluminescent substrate AMPPD. The resultant signals produced can be detected using standard X-ray film or a phosphoimager in as little as 30 minutes. Light continues to be given off for up to 48 hours for longer developments.

#### ***The DIG System in Thermo Hybridisation Ovens***

The small probe volumes, even mixing, accurate temperature control and ease of handling associated with Thermo Oven systems are equally suited to the success of non-radioactive Hybridisation.

The protocol detailed below has been developed in conjunction with the Böehringer DIG system. The prehybridisation, Hybridisation and washing stages of the protocol using the DIG labelled probe are exactly as for radio-labelled probes. The subsequent wash stages associated with detection and visualisation of the probe is often performed in sandwich boxes, but will work equally well in bottles.

This protocol has been optimised for DNA RFLP analysis. It may require minor alteration to be used for other applications.

The following protocol applies to a 20cm x 20cm membrane: -

## ***Electrophoresis & Transfer***

Electrophorese and transfer DNA fragments on to positively charged Hybridisation membrane (Böehringer Mannheim, Cat N° 1209 272). Bake at 120°C for 30 minutes to fix DNA or UV cross-link.

### ***DNA Hybridisation***

Prehybridisation and Hybridisation are carried out in bottles in a Thermo rotisserie oven. Better results have been obtained in bottles than in bags.

1. Pre-warm Hybridisation bottle containing 50ml 2 x SSC to 68°C.
2. Layer the following into a plastic box containing 50ml 2 x SSC:
  - a) One piece of nylon mesh (23cm x 23cm - Thermo Scientific).
  - b) The membrane, DNA side-up.
  - c) Two layers of nylon mesh.
  - d) One piece of 'dummy' Hybridisation membrane covering Hybridisation membrane below.
  - e) One piece of nylon mesh.

This procedure prevents high backgrounds. (Mesh and 'dummy' membrane are reusable after washing in distilled water.)

3. Roll 'sandwich' (ensuring no air bubbles are trapped) with DNA side facing inwards.
4. Place roll in Hybridisation bottle and carefully unroll 'sandwich' again ensuring no air bubbles are trapped.
5. Tip off 2 x SSC and add 20ml prehybridisation buffer pre-warmed to 68°C.
6. Prehybridise in rotisserie oven for 2 hours at 68°C.
7. Denature 300ng DIG labelled probe. Add to 15ml-prehybridisation buffer heated to 68°C.
8. Tip buffer off prehybridised membrane and add Hybridisation buffer to the bottle. Hybridise overnight in rotisserie oven at 68°C.
9. Remove Hybridisation buffer and freeze. This can be reused a further five times after heating to 95°C for 10 minutes.
10. Add 50ml 2 x SSC 0.1% SDS to bottle and roll in opposite direction to release 'sandwich'.

11. Perform washes in bottle replacing each wash with: -

|                |                       |                  |
|----------------|-----------------------|------------------|
| 2 x 5 minutes  | in 50-100ml 4 x SSC   | 1% SDS at 68°C   |
| 2 x 15 minutes | in 50-100ml 2 x SSC   | 0.1% SDS at 68°C |
| 1 x 15 minutes | in 50-100ml 0.1 x SSC | 0.1% SDS at 68°C |

Alternatively, remove membrane from roll and wash in plastic box: -

|                |                    |                        |
|----------------|--------------------|------------------------|
| 2 x 5 minutes  | in 500ml 2 x SSC   | 0.1% SDS at room temp. |
| 2 x 15 minutes | in 500ml 0.2 x SSC | 0.1% SDS at 68°C       |

## CHAPTER 6

### HYBRIDISATION GUIDE

#### HYBRIDISATION USING OLIGONUCLEOTIDE PROBES

---

The Hybridisation conditions outlined in Chapter 3 are generally applicable to probes derived by cloning, or by gene amplification reactions ranging in size from 100bp to several hundred base pairs. The Hybridisation conditions of synthetic oligonucleotide probes, however, are rather more problematical.

There are two types of oligonucleotide probes: -

1. A single oligonucleotide of a defined sequence, derived from the nucleic acid sequence of the target gene.
2. A pool of oligonucleotides of degenerate sequences, which are derived from the amino acid sequence of a region of the gene product of interest. Due to the degeneracy of the genetic code (i.e. most amino acids are specified by more than one triplet codon) any particular sequence of amino acids will be specified by a number of different oligonucleotides.

Because the  $T_m$  of synthetic oligonucleotides is much lower than for longer probes, the stringency of Hybridisation and washing procedures must be reduced and adjusted according to the base composition of the probe. An approximate value for the  $T_m$  of an oligonucleotide probe has been derived by Wallace *et al* (1979): -

$$T_m (^{\circ}\text{C}) = 2 \times (\text{number of AT base pairs}) + 4 \times (\text{numbers of GC base pairs}).$$

Hybridisations are carried out at 5°C below this temperature. For a degenerate pool of oligonucleotides, Hybridisation is carried out at 5°C below the lowest  $T_m$  for the set of sequences.

1. Prehybridise the membrane in oligonucleotide prehybridisation buffer (6 x SSPE (SSC), 10 x Denhardt's reagent, 50µg/ml denatured salmon sperm DNA) at the Hybridisation temperature for at least one hour.
2. Add the radio-labelled probe solution to the preHybridisation buffer. Alternatively, remove a small amount of preHybridisation buffer, add the probe to this and replace this solution in the Hybridisation vessel. Some researchers may prefer to use fresh Hybridisation buffer.
3. Hybridise with agitation, or by rotating in bottles, for approximately 12 hours at the Hybridisation temperature.

4. Stringency washing is carried out as follows using large volumes (at least 50ml) of the following solutions, pre-warmed to the required temperature:

|                |                     |                                     |
|----------------|---------------------|-------------------------------------|
| 2 x 15 minutes | with 6 x SSPE (SSC) | 0.1% SDS at the Hybridisation temp. |
| 1 x 2 minutes  | with 6 x SSPE (SSC) | 0.1% SDS at the T <sub>m</sub>      |

5. Wrap the membrane in Saran Wrap™ and autoradiograph at -70°C in a cassette with an intensifying screen. Expose initially for approximately 12 hours (or overnight).

Background Hybridisation problems are much more common when using oligonucleotide probes. For this reason procedures to reduce background such as pre-washing of colony filters (Chapter 2, Colony/Plaque Blotting) and purification of the oligonucleotide to remove unincorporated nucleotides are particularly advantageous.

## CHAPTER 7

### HYBRIDISATION GUIDE

#### TROUBLESHOOTING GUIDE FOR NUCLEIC ACID HYBRIDISATIONS

---

Our bottle system is intrinsically simpler and safer to use than other methods of Hybridisation such as Hybridisation in bags or plastic boxes. In Hybridisation Ovens, the temperature of the solutions is precisely controlled and regulated and, in addition, the rotisserie device ensures that the solutions are continuously and evenly distributed over the membrane. Thus, the optimum conditions for Hybridisation and washing are maintained throughout when using this system. However, during the transfer of protocols from bags to bottles some minor adjustments to the protocols may be necessary.

When loading the mesh and membranes into the bottles, air bubbles should be avoided. Ensure that the Oven is sited on a flat surface so that the probe solution is distributed evenly along the length of the bottles and that there is sufficient probe solution to cover the entire membrane. On occasions the mesh and membrane can become tightly rolled up in the bottle. This occurs if the mesh is loaded incorrectly (*see Figure 3 in Chapter 3*).

#### ***Background Reduction - General***

All solutions for nucleic acid Hybridisations should be prepared using distilled water and highest quality reagents in clean glassware. In particular, water with a high organic content will cause bad background problems. Formamide should be freshly de-ionised. Membranes should always be handled wearing gloves or with forceps. The following should also be taken into consideration: -

#### ***Prehybridisation Procedure***

Prehybridisation is required to block the sites on the nylon membrane, which the probe would otherwise bind to non-specifically. Failure to carry out adequate preHybridisation results in high backgrounds. If dextran sulphate is used in the Hybridisation solution, then it must also be included in the prehybridisation solution.

## ***Washing Procedure***

Stringency washing should be carried out as follows, using large volumes (approximately 100ml) of the following solutions which should be pre-warmed to the required temperature: -

1. 2 x 15 minutes with 2 x SSPE (SSC), 0.1% SDS at 65°C
2. 1 x 30 minutes with 2 x SSPE (SSC), 0.1% SDS at 65°C
3. 1 x 10 minutes with 0.1 x SSPE (SSC), 0.1% SDS at 65°C

All wash solutions should be pre-warmed to the appropriate temperature. An initial room temperature wash is not recommended and can cause background problems.

The final wash is a high stringency wash. Use of a hand held monitor to give an indication of the decrease in radioactivity as the washes progress is recommended and should give some indication as to whether this final wash should be carried out.

In general terms, the stringency of Hybridisation and washing steps is increased by increasing the temperature, or by decreasing the salt concentration. Hybridisation should be carried out under relatively low stringency conditions compared to the washing procedures. It is generally simpler and more effective to adjust the stringency during the washing steps by altering the salt concentration rather than the temperature.

## ***Probe Preparation***

The final probe concentration should be in the region of 25-50ng/ml of Hybridisation solution, at approximately  $1-5 \times 10^6$ cpm/ml.

The optimum length of probe is approximately 500-800bp. Purification of the labelled probe to remove unreacted triphosphates will reduce background problems, and is recommended for all Hybridisations - Thermo Recovery kits are excellent for this purpose. Probe solutions should be pre-warmed to the Hybridisation temperature and care should be taken to ensure the membrane is not exposed to the concentrated probe solutions if adding it directly to the bottles.

## Summary of Factors Resulting in High Backgrounds

1. Hybridisation solutions and/or wash solutions not pre-warmed before use.
2. Probe concentrations too high or probe not denatured. When transferring Hybridisation protocols to bottles the volumes will be reduced. Ensure that probe concentrations are adjusted accordingly.
3. Unincorporated nucleotides not removed from probe solution.
4. Insufficient prehybridisation or blocking agents in prehybridisation and Hybridisation solutions (e.g. Denhardt's reagent and salmon sperm DNA). An adequate prehybridisation is important to block non-specific Hybridisation to the membrane.
5. Hybridisation and/or washing conditions not stringent enough: -
  - (i) Decrease salt concentration.
  - (ii) Increase temperature.
  - (iii) Increase concentration of SDS.
  - (iv) Increase wash times.
6. Membranes drying out. This may often be the cause of an apparent overlap problem and may result from: -
  - (i) Too low a probe volume.
  - (ii) Too slow a change over of solutions, particularly when bulk processing.
  - (iii) Oven not level.
  - (iv) Excessive variable axis angle.
7. Residual agarose on membranes may cause foggy backgrounds. Membranes should be rinsed in 2 x SSC to remove residual agarose and excess salt after blotting and prior to fixing (especially following vacuum blotting).
8. Multiple filters not separated by mesh in bottles.
9. Autoradiography problems. Random black spots and "lightening flash" markings on autoradiographs may be due to static electricity.

## Summary of Factors Resulting in a Signal Lower than Expected

1. Insufficient exposure time of membrane to film during autoradiography or phosphoimaging.
2. Inefficient transfer and/or binding of nucleic acids to the nylon membrane.
3. Target sequence present at very low copy numbers. Increase the amount of sample loaded on to the gel.
4. Probe sequence not present in sufficient quantities. Increase the concentration of probe or include 10% dextran sulphate, which reduces the solvent volume and has the same effect.
5. No probe homology.
6. Double-stranded DNA probe was not denatured - see standard protocols. Alternatively, probe degraded. This is more likely to occur when using RNA probes.
7. The specific activity of the probe was too low. Consider factors such as the probe concentration during the labelling reaction, half-life of radio-labelled triphosphates, etc.
8. Hybridisation and/or washing conditions were too stringent: -
  - (i) Increase salt concentration.
  - (ii) Decrease temperature.
  - (iii) Reduce concentration of SDS.
  - (iv) Reduce wash times.
9. The Hybridisation time was too short.

## APPENDIX I

### SOLUTIONS FOR NUCLEIC ACID BLOTTING & HYBRIDISATION PROCEDURES

---

**1. 20 x SSC (1 litre)**

|                |      |
|----------------|------|
| NaCl           | 175g |
| Sodium citrate | 88g  |

Dissolve in 800ml of H<sub>2</sub>O and adjust pH to 7.0 with NaOH solution. Adjust the volume to 1 litre with H<sub>2</sub>O and sterilise by autoclaving.

**2. 20 x SSPE (1 litre)**

|                                                   |       |
|---------------------------------------------------|-------|
| NaCl                                              | 175g  |
| NaH <sub>2</sub> PO <sub>4</sub> H <sub>2</sub> O | 27.6g |
| Na <sub>2</sub> EDTA                              | 7.4g  |

Dissolve in 800ml of H<sub>2</sub>O and adjust pH to 7.4 with NaOH solution. Adjust the volume to 1 litre with H<sub>2</sub>O and sterilise by autoclaving.

**3. 100 x Denhardt's Reagent**

|                                   |    |
|-----------------------------------|----|
| Ficoll                            | 2g |
| Polyvinyl pyrrolidone             | 2g |
| Bovine serum albumin (Fraction V) | 2g |

Dissolve in 100ml H<sub>2</sub>O, filter and store in aliquots at -20°C.

**4. Salmon Sperm DNA**

Dissolve the DNA at a concentration of 1mg/ml in sterile H<sub>2</sub>O, sonicate to a length of 200-500bp, and then stand in a boiling water bath for 10 minutes. Aliquot and store at -20°C.

**5. Denaturing Solution**

|      |      |
|------|------|
| NaCl | 1.5M |
| NaOH | 0.5M |

Sterilise by autoclaving.

## 6. Neutralising Solutions

|                      |        |
|----------------------|--------|
| NaCl                 | 1.5M   |
| Tris Cl pH7.2        | 0.5M   |
| Na <sub>2</sub> EDTA | 0.001M |

Sterilise by autoclaving.

## 7. 10% Sodium Dodecyl Sulphate (SDS)

(Also known as sodium lauryl sulphate.)

## 8. Formamide

Formamide should be de-ionised before use. Add 5g of a mixed bed ion exchange resin (e.g. Biorad AG501) to 100ml formamide and stir for 1-2 hours. Store at -20°C.

## 9. Pre-wash Solution

5 x SSC  
0.5% SDS  
1mM Na<sub>2</sub>EDTA

## 10. Prehybridisation Buffer for Southern Blots

Prepare as follows (10ml):

| <b><i>Solution</i></b>   | <b><i>Volume</i></b> | <b><i>Final Concentration</i></b> |
|--------------------------|----------------------|-----------------------------------|
| 20 x SSPE (or SSC)       | 3ml                  | 6 x SSPE (SSC)                    |
| 100 x Denhardt's reagent | 0.5ml                | 5 x Denhardt's                    |
| 10% SDS                  | 0.5ml                | 0.5% SDS                          |
| H <sub>2</sub> O         | 4.5ml                |                                   |

Add denatured salmon sperm DNA to 50µg/ml.

## 11. Prehybridisation Buffer for Northern Blots

Prepare as follows (10ml):

| <b><i>Solution</i></b>   | <b><i>Volume</i></b> | <b><i>Final<br/>Concentration</i></b> |
|--------------------------|----------------------|---------------------------------------|
| 20 x SSPE                | 2.5ml                | 5 x SSPE                              |
| Formamide                | 5ml                  | 50%                                   |
| 100 x Denhardt's reagent | 0.5ml                | 5 x Denhardt's                        |
| 10% SDS                  | 0.5ml                | 0.5% SDS                              |
| H <sub>2</sub> O         | 1.5ml                |                                       |

Add denatured salmon sperm DNA to 100µg/ml.

**NB:** All solutions should be prepared in clean, sterile glassware using distilled water and highest quality reagents.

**NB:** In addition, Hybridisation membranes should always be handled wearing gloves or using forceps. Where it is necessary to sterilise the membranes, place between sheets of filter paper and autoclave for 15 minutes.

## APPENDIX II

### FACTORS AFFECTING STRINGENCY OF HYBRIDISATION REACTIONS

The stringency of Hybridisation determines the degree to which mismatched hybrids are permitted to form. The optimum Hybridisation temperature for DNA - DNA Hybridisations is 25°C below the melting temperature (T<sub>m</sub>).

The T<sub>m</sub> is affected by: -

1. The concentration of monovalent cation (M).
2. Base composition (percentage G+C content).
3. The length of the duplex in base pairs (L).
4. The amount of formamide in the reaction mixture (F).

These variables have been linked by the following equation (see Meinkoth and Wahl, 1984): -

$$T_m = 81.5^{\circ}\text{C} + 16.6 \log M + 0.41 (\% \text{ G+C}) - 0.61 (\% \text{ F}) - 500/L$$

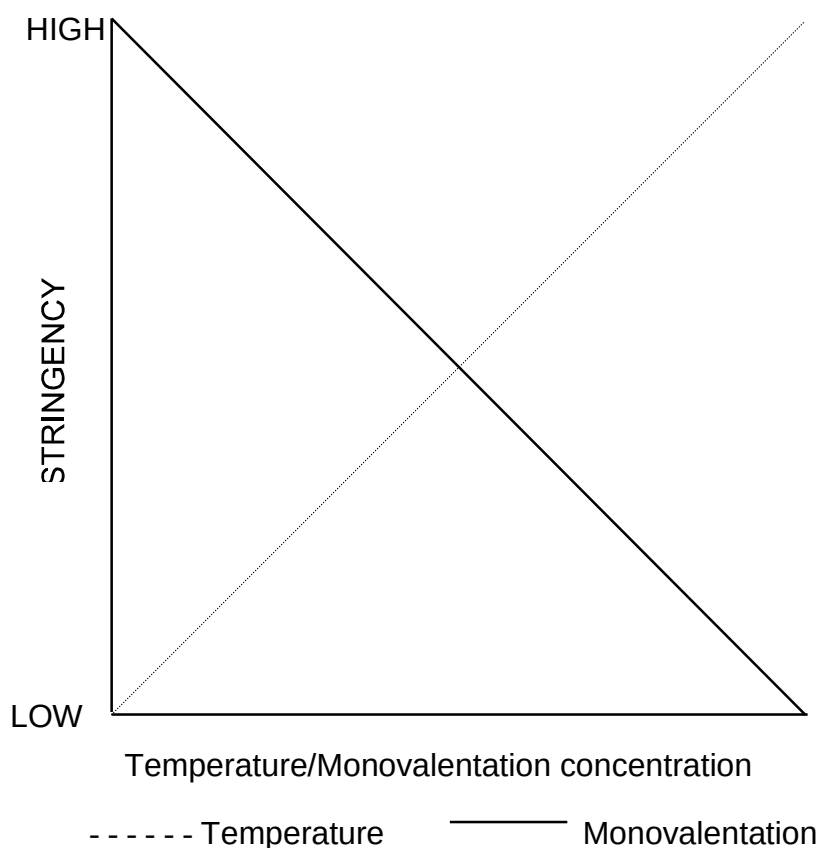
This equation relates to probes longer than approximately 50 nucleotides (see Chapter 6 for conditions relating to oligonucleotide Hybridisations).

As an illustration, in a reaction carried out in a solution of 6 x SSC and no formamide, with a 50% GC rich 500bp probe, the T<sub>m</sub> is calculated to be 101°C (i.e. Hybridisation temperature 75°C).

Substituting the 6 x SSC with 1 x SSX in this system (i.e. during washing), reduces the T<sub>m</sub> by 13°C. Including 50% formamide in each solution would have the effect of reducing each T<sub>m</sub> by 30.5°C. A shorter probe length (50bp) reduces the T<sub>m</sub> by a further 10°C.

In practice, it is not necessary to calculate the T<sub>m</sub> accurately for every system, and the standard conditions given in the protocol sections will give good results. However, the equation does give a useful indication of the effect of adjusting the different variables on the stringency. In general, hybridise at low stringency; then during washing, increase the stringency by keeping the temperature constant but decreasing the salt concentration with each wash step.

## ***Effect of Temperature & Salt Concentration***



## ***Summary of Conditions Affecting Hybridisation***

### ***Temperature***

The Hybridisation temperature is one of the most critical factors because it affects the rate of hydrogen bond formation between DNA strands; the hydrogen bonds in effect overcome the repulsive forces between the negatively charged strands. If the temperature is increased, then the stringency of Hybridisation is increased.

### ***Ionic Strength***

Increasing the monovalent cation concentration enables the DNA strands to come together more readily, thus reducing the stringency of Hybridisation. EDTA is sometimes added to chelate magnesium ions, which can have an unpredictable effect on Hybridisation reactions.

## ***Base Composition***

Three hydrogen bonds are formed during G-C base pairing. G-C rich probes bind more efficiently, but may tend to bind non-specifically. Therefore, probes should have a G-C content of approximately 50-60%.

## ***Formamide***

Formamide acts as a destabilising agent, which reduces hydrogen bond formation. Hybridisation reactions using formamide are therefore performed at lower temperatures.

## ***Probe Length & Complexity***

A long complex probe will hybridise more slowly than a short probe.

## ***High Molecular Weight Polymers, e.g. Dextran Sulphate***

Dextran sulphate acts as an accelerator. In effect, it excludes the probe itself from a large volume of the probe solution effectively increasing local probe concentration. It also acts to some degree as a blocking agent.

## ***Blocking Agents***

For example, sonicated salmon sperm DNA, Denhardt's reagent. Blocking agents act as analogues to the probe, binding to regions in the filter that the DNA would otherwise tend to bind to non-specifically.

## ***Detergents***

Sodium dodecyl sulphate has the effect of increasing the stringency of Hybridisation, and can act as a blocking agent.

## ***pH***

For optimum results the pH of the Hybridisation solution should be maintained between pH 5-9.

## ***Tetramethyl Ammonium Chloride (TMAC)***

Generally speaking, A-T base pairs are less stable than G-C base pairs. TMAC, at a concentration of 3M, shifts the melting temperature of A-T base pairs to that of G-C base pairs. Thus it may be used in oligonucleotide Hybridisation so that the stringency of Hybridisation becomes a function of the probe length alone, rather than probe length and G-C content.

## APPENDIX III

### HYBRIDISATION BOTTLE SAFETY

---

#### ***Use of the Bottle Gripper***

(Catalogue No HB-OV-BGS - to fit 15cm, small bottles)

(Catalogue No HB-OV-BGL - to fit 30cm, medium & large bottles)

(Catalogue No HB-OV-BGXL - to fit 30cm, extra large bottles)

This silicone rubber safety sleeve fits neatly over all our standard bottles and is included with every Thermo Oven we ship.

- Helping you to safely tighten and unscrew caps.
- Making handling of hot bottles easier.

For more details and ordering information, contact Thermo Scientific or your local Distributor.

#### ***Bottle Care***

Hybridisation Bottles are made of thick walled borosilicate glass, which protects users from radiation and has excellent long-term reliability.

- ☑ It is important to check your bottles regularly for chips, stress fractures and cracks. If these occur, the bottle must be discarded.
- ☑ Ensure bottles are stored either in a suitable rack or with caps replaced in between experiments. This will protect the bottle and sealing area.
- ☑ Replace “O” rings when worn or leaky. Replace all “O” rings every six months.
- ☑ Wear protective gloves to protect your hands in the event of accidental breakage.
- ☑ Never over tighten caps on bottles. Hand tight only is sufficient.
- ☑ If the bottle cap is difficult to unscrew **NEVER ATTEMPT** to force the bottle cap open. Allow the bottle to cool and retry. If the cap remains stuck, discard the bottle.
- ☑ The bottles should not be used at temperatures above 70°C.

**NB:** Extra large bottles (70mm diameter) are supplied with a vented cap to prevent excessive pressure build-up during use. A 0.45µm hydrophobic tissue culture filter should be fitted to the vent outside the bottle as an added safety measure.

## APPENDIX IV

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